PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07K 7/23, A61K 47/48 — A1

(11) International Publication Number: WO 97/19954

(43) International Publication Date: 5 June 1997 (05.06.97)

(21) International Application Number: PCT/EP96/05029

(22) International Filing Date: 14 November 1996 (14.11.96)

(30) Priority Data:
08/562,652 27 November 1995 (27.11.95) US

(71) Applicant: ASTA MEDICA AKTIENGESELLSCHAFT [DE/DE]; An der Pikardie 10, D-01277 Dresden (DE).

(72) Inventors: SCHALLY, Andrew, V.; Tulane University Medical Center, School of Medicine, 1430 Tulane Avenue, New Orleans, LA 70112-2699 (US). NAGY, Attila, A.; Tulane University Medical Center, School of Medicine, 1430 Tulane Avenue, New Orleans, LA 70112-2699 (US). CAI, Ren-Zhi; Tulane University Medical Center, School of Medicine, 1430 Tulane Avenue, New Orleans, LA 70112-2699 (US).

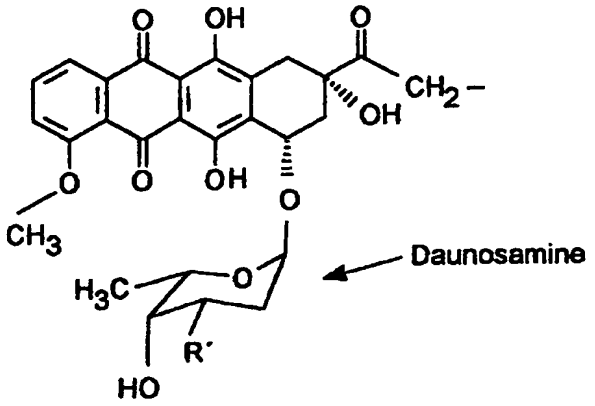
(81) Designated States: AU, BR, CA, CN, CZ, HU, IS, JP, KR, MX, NO, NZ, PL, SK, UA, Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: TARGETED CYTOTOXIC ANTHRACYCLINE ANALOGS

(57) Abstract

This invention is in the field of the chemistry of targeting anticancer anthracycline derivatives. More particularly, it concerns doxorubicin (DOX) or its daunosamine modified derivatives (DM-DOX) linked covalently to analogs of peptide hormones such as LH-RH, bombesin and somatostatin. These covalent conjugates are targeted to various tumors bearing receptors for the peptide hormone analogs. The compounds of this invention are represented



(II)

by the General Formula: Q^{14}-O-R-P wherein Q has general formula (II) wherein: Q^{14} signifies a Q moiety with a side chain at the 14 position; R- is H or -C(O)-$(CH_2)_n$-C(O)- and n = 0-7; R' is NH_2 or an aromatic, saturated or partially saturated 5- or 6-membered heterocyclic compound having at least one ring nitrogen and optionally having a butadiene moiety bonded to adjacent carbon atoms of said ring to form a bicyclic system; P is H or a peptide moiety, suitably an LHRH, somatostatin or bombesin analogs. Nevertheless where R' is NH_2, then R and P are other than H. When R and P are H, then R' is other than NH_2. A novel synthetic reaction has been discovered in the course of this work to form partially saturated heterocyclic moieties from vicinal and disjunct i.e., α,β- or α,γ-hydroxy primary amines. Q_1 is DOX; Q_2 is 3'-deamino-3'-(pyrrolidine-1''-yl)-doxorubicin (AN 181); Q_3 is 3'-deamino-3'-(isoindoline-1''-yl)-doxorubicin (AN 184); Q_4 is 3'-deamino-3'-(3''-pyrroline-1''-yl)-doxorubicin (AN 185); Q_5 is 3'-deamino-3'-(3''-pyrrolidone-1''-yl)-doxorubicin (AN 191); Q_6 is 3'-deamino-3'-(2''-pyrroline-1''-yl)-doxorubicin (AN 201); Q_7 is 3'-deamino-3'-(3''-piperidone-1''-yl)-doxorubicin (AN 195); Q_8 is 3'-deamino-3'-(1'',3''-tetrahydropyridine-1''-yl)-doxorubicin (AN 205). $Q_1{}^{14}$gL is AN 152; $Q_6{}^{14}$gL is AN 207; $Q_1{}^{14}$gS is AN 162; $Q_6{}^{14}$gS is AN 238; $Q_1{}^{14}$gB is AN 160; $Q_6{}^{14}$gB is AN 215.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgystan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic			SE	Sweden
CG	Congo	KR	Republic of Korea	SG	Singapore
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LR	Liberia	SZ	Swaziland
CS	Czechoslovakia	LT	Lithuania	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	LV	Latvia	TJ	Tajikistan
DK	Denmark	MC	Monaco	TT	Trinidad and Tobago
EE	Estonia	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	UG	Uganda
FI	Finland	ML	Mali	US	United States of America
FR	France	MN	Mongolia	UZ	Uzbekistan
GA	Gabon	MR	Mauritania	VN	Viet Nam

TARGETED CYTOTOXIC ANTHRACYCLINE ANALOGS

Background of the Invention

This invention was made in part with Government support. The Government has certain rights in this application.

Field of the Invention

This invention is in the field of the chemistry of targeting anticancer anthracycline derivatives. More particularly, it concerns doxorubicin (DOX) or its daunosamine modified derivatives (DM-DOX) linked covalently to analogs of peptide hormones such as LH-RH, bombesin and somatostatin. These covalent conjugates are targeted to various tumors bearing receptors for the peptide hormone analogs.

Discussion of the Prior Art

LH-RH Analogs which have cytotoxic moieties at the sixth position are shown in Schally, Janaky and Bajusz, EP 0 450 461 B1, grant publication September 6, 1995.

GnRH (LH-RH) analogs for destroying gonadotrops are described in Nett and Glode, WO 90/09799, published on September 7, 1990. This application describes toxins, like ricin, linked to analogs of LH-RH for destroying gonadotrophs and thus curing sex hormone dependent cancers. LH-RH doxorubicin derivative is also mentioned without specification of the chemistry of linking.

Cytotoxic somatostatin analogs are described by Schally et al. in U.S. Pat. application filed on April 6, 1990 and refiled in July 15, 1993 under Serial No. 08/076,846

A review by A. V. Schally in Anti-Cancer Drugs 5, 115-130 (1994) gives details about the presence of receptors on the cell membranes of a wide variety of tumors for analogs of LH-RH, bombesin or somatostatin.

G. Weckbecker lists several references that show the presence of receptors and receptor subtypes for somatostatin analogs on several normal and tumorous tissues in his review in Farmac. Ther. 60, 245-264 (1993).

Bombesin-like peptides and the presence of bombesin/GRP receptors on various normal and tumorous tissues are discussed in the review by N. Bunnett in Gut Peptides: Biochemistry and Physiology 423-445 (1994) Ed.: J. Walsh and G. J. Dockray, Raven Press, New York and by E. Spindell in Recent Progress in Hormone Research 48, (1993) (Academic Press)

Doxorubicin (DOX) is, at this time, the most widely used, and very potent anticancer agent. However, certain tumors do not respond to it at all and its use is also limited by multidrug resistance (MDR) and cardiotoxicity as well as neutropenia, which are the results of chronic treatment. In order to overcome these drawbacks and to further exploit the enormous tumoricidal potential inherent in the structure of anthracycline antibiotics, thousands of synthetic derivatives have been described, including their targeted analogs linked to various carrier macromolecules.

Most of the history of DOX and its analogs is described in "Adriamycin", David W. Henry, ACS Symposium Series, No. 30, Cancer Chemotherapy, American Chemical Society, pp. 15-57 (1976) and in the book Doxorubicin, Federico Arcamone, Academic Press, (1981).

Highly active, alkylating, non-cross resistant 3'-deamino-3'-(3"-cyano-4"-morpholinyl)-DOX and derivatives thereof which have antitumor activity are described in Mosher, et al., U.S. Pat. 4,464,529, August 7, 1984. The synthesis and biological evaluation of these "Intensely Potent Morpholinyl Anthracyclines" are also described in J. Med. Chem. 1984, 27, 638-645.

In Proc. Natl. Acad. Sci. USA Vol. 88, pp. 4845-4849, June 1991. Gao et al. describe formaldehyde-mediated alkylation of a DNA sequence by a daunorubicin derivative.

Anthracycline analogues bearing latent alkylating substituents are described in J. Med. Chem. 35, 3208-3214 (1992).

The use of an α,ω-diiodo compound for the alkylation of the daunosamine nitrogen of DOX and thus the formation of a new morpholinyl DOX derivative is described in European Patent EP 434 960, filed by Pharmacia Carlo Erba on December 12, 1989.

N-Trifluoroacetyladriamycin^{14}-O-hemiglutarate and -hemiadipate are disclosed as analogs of N-trifluoroacetyladriamicyn^{14}-O-valerate (AD-32) with improved water solubility in Israel, et al., U.S. Patent 4,299,822, November. 10, 1981.

Horton and Priebe (J. Antibiotics, XXXVI, 1211-1215.) describe several 14-O-esters of different anthracycline analogs with no dramatic changes in anticancer activity as compared to the 14-OH parent analogs.

In the art of designing targeted chemotherapeutic agents, the following objectives are sought:

1. Stable linkage between the carrier molecule and the chemo-therapeutic agent until the target is reached.
2. Retained biological characteristics of the carrier molecule within the conjugate, such as retained binding properties.
3. Retained pharmacological activity of the chemotherapeutic agent within the conjugate, such as retained cytotoxic activity.
4. As a result of conjugation, the production of analogs of more intense activity and/or lower peripheral toxicity relative to the unconjugated moieties.

Conjugation of DOX by $NaIO_4$ oxidation of the daunosamine moiety of DOX followed by reductive alkylation involving a primary amine of a carrier molecule is described in Sela, et al., U.S. Patent 4,263,279, April 21, 1981.

A cis-aconitic acid spacer was used to link the daunosamine nitrogen to macromolecular carriers with a pH-sensitive bond, as described in Biochem. Biophys. Res. Commun. 1981 102, 1048-1054.

The formation of ester bonds and C-N linkages between 14-bromodaunorubicin and proteins or poly-L-amino acids is described by Zunino et.al. (1981) Tumori 67, 521-524 and (1984) Eur. J. Cancer Clin. Oncol. 20, 421-425.

Morpholino-DOX (a highly active, daunosamine modified analog of DOX) was conjugated to antibody via a hydrolyzable (lysosomotrop, pH sensitive) hydrazone linkage, involving the C-13 oxo function of the cytotoxic agent, as described in Bioconjugate Chemistry 1990 1(5), 325-330

Sensitivity of the carboxamide bond of a leucine residue to enzymatic degradation was used successfully in conjugates of DOX containing a "spacer arm" peptide, preferentially Ala-Leu-Ala-Leu, where the carboxy terminal Leu acylates the daunosamine nitrogen in DOX and the amino

terminal Ala is linked to the carrier through dicarboxylic acid spacer as described in Proc. Natl. Acad. Sci. USA 1982 79, 626-629.

The daunosamine nitrogen of DOX was acylated by a glutaric acid spacer and linked to LH-RH analogs with a severe loss of cytotoxic activity as described in Proc. Natl. Acad. Sci. USA 1992 89, 972-976.

Further references related to the use of the compounds according to the present invention for the treatment of various human tumors:

1. Schally et.al. (1996) in Treatment with GnRH Analogs: Controversies and Perspectives, eds. Filicori, M. & Flamigni, C. (Parthenon,Carnforth, U.K.), pp. 33-44.

2. Nagy et.al.(1996) Proc. Natl. Acad. Sci. U.S.A. 93, 7269-7273.

3.Yano et.al. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 7090-7094.

4. Rekasi et.al. (1993) Endocrinology 132(5) 1991-2000.

5. Srkalovic et.al. (1990) Cancer Res. 50, 1841-1846.

6. Emons et.al. (1993) Cancer Res. 53, 5439-5446.

7. Emons et.al. (1993) Journal of Clin. Endocrin. and Metabol. 77(6) 1458-)

8. Schally, A. V. (1988) Oncological applications of somatostatin analogs. Cancer Res. 48, 6977-6985.

9. Schally et.al. (1994) International Journal of Pancreatology 16, 277-280.

10. Srkalovic et.al. (1990) Journal of Clinical Endocrinology and Metabolism 70(3), 661-669. 4 Pinski et.al. (1994) Int. J. Cancer 57, 574-580.

11. Radulovic et.al. (1992) Cancer Letters 62, 263-271.

12. Qin et.al. (1995) Int. J. Cancer 60, 694-700.

13. Radulovic et.al. (1992) P.S.E.B.M. 200, 394-401.

14. Radulovic et.al. (1994) Acta Oncologica 33(6) 693-701.

15. Pinski et.al. (1993) Cancer Letters 71, 189-196.

16. O'Byrne et.al. (1994) Eur. J. of Cancer 30A(11) 1682-1687.

17. Pinski et.al. (1994) Br. J. of Cancer 70, 886-892.

18. Pinski et. al. (1994) Cancer Res. 54, 5895-5901.

19. Pinski et.al. (1996) Int. J. Cancer 65, 870-874.
20. Banks et.al. (1992) Anticancer Drugs. 3, 519-523.
21. Reubi and Kvols (1992) Cancer Res. 52, 6074-6078.)
22.Schally et.al. (1994) International Journal of Pancreatology 16, 277-280.
23.Halmos et.al. (1995) Cancer Res. 55, 280-287.
24. Halmos et.al. (1994) Cancer Letters 85, 111-118.
25. Qin et.al. (1994) J. Cancer Res. Clin. Oncol. 120, 519-528
26. Qin et.al. (19940 Cancer Res. 54, 1035-1041.
27. Qin et.al. (1995) Int. J. Cancer 63, 257-262.
28. Reile et.al. (1994) The Prostate 25, 29-38.
29. Pinski et.al. (1994) Int. J. Cancer 57, 574-580.
30. Radulovic et.al. (1992) P.S.E.B.M. 200, 394-401.
31. Radulovic et.al. (1994) Acta Oncologica 33(6) 693-701.
32. Pinski et.al. (1993) Cancer Letters 71, 189-196.
33. Pinski et.al. (1994) Br. J. of Cancer 70, 886-892.
34. Pinski et. al. (1994) Cancer Res. 54, 5895-5901.)

All the citations referred to are incorporated herein by reference.

Summary of the Invention

The compounds of the invention are novel, targeted cytotoxic peptide hormones comprising an anthracycline cytotoxic agent, such as DOX or DM-DOX, conjugated to a peptide hormone, such as analogs of LH-RH, bombesin, and somatostatin. These cytotoxic peptide hormone conjugates are designed for the treatment of tumors bearing specific receptors for the conjugate, such as breast cancer, ovarian, cancer, endometrial cancer, prostate cancer, pancreatic cancer, colon cancer, gastric cancer, and lung cancer. Certain of these (unconjugated) anthracycline cytotoxic agents utilized herein are per se novel, and are highly potent, their level of toxicity however is too high for them to be used in unconjugated form.

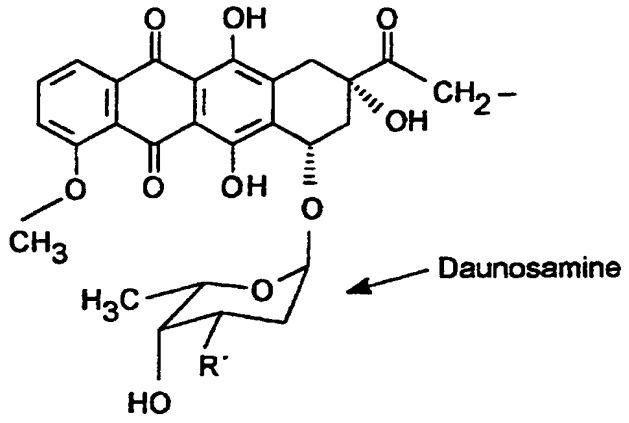
Daunosamine modified DOX analogs presented in this invention were developed during a search for new, highly active, non-cross resistant analogs of DOX suitable for the formation of covalent conjugates with peptide carriers.

The formation of stable, covalently linked conjugates with fully retained biological activities of their components was achieved by using a dicarboxylic acid spacer, like glutaric acid. One carboxyl group of the spacer forms an ester bond with the 14-OH group of DOX or DM-DOX and the other carboxyl group of the spacer forms a carboxamide bond with a well chosen free amino group of the peptide carrier.

The compounds of this invention are represented by General Formula

$$Q^{14}\text{-}O\text{-}R\text{-}P \qquad (I)$$

wherein Q has the general formula



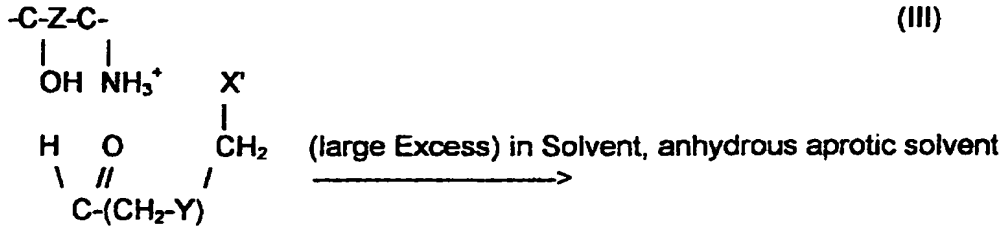
(II)

Q^{14} signifies a Q moiety with a side chain at the 14 position,

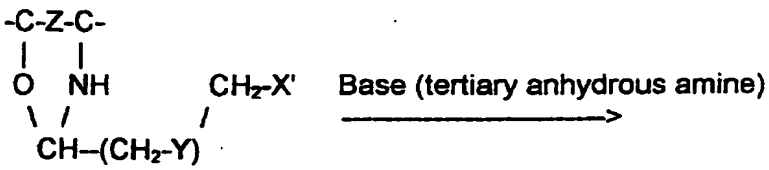
R- is H or -C(O)-$(CH_2)_n$-C(O)- and n=0-7,
R' is NH_2 or an aromatic, saturated or partially saturated 5 or 6 membered heterocyclic compounds having at least one ring nitrogen and optionally having a butadiene moiety bonded to adjacent carbon atoms of said ring to form a bicyclic system,
P is H or a peptide moiety, suitably an LHRH, somatostatin or bombesin analog, but not excluding other physiologically active peptides. Particularly desirable are those LHRH analogs having affinity for neoplastic cell receptors, especially those analogs having a D-Lys moiety at the 6 position, as well as shortened somatostatin and bombesin analogs. Nevertheless where R' is NH_2 then R and P are other than H. When R and P are H, then R' is other than NH_2.

A novel synthetic reaction has been discovered in the course of this work. Not only was it found that doxorubicin and its derivatives can be coupled via a dicarboxylic moiety at the 14 position to yield novel pharmacologically effective conjugates but a novel way was provided to form partially saturated heterocyclic moieties from vicinal and disjunct i.e. α,β- or α,γ-hydroxy primary amines. The particular application in the present invention was the formation of 2"-pyrrolinyl and 1",3"- tetrahydropyridinyl moieties on the daunosamine sugar. However, this reaction has broader applicability. 5 and 6 membered partially saturated heterocyclic moieties may be formed when a vicinal or disjunct hydroxy amine is reacted with a halo-substitued aldehyde having 2 or 3 moieties between the aldehyde carbon and the carbon atom having the halo group. These moieties may all be methylene, or a hetero atom such as oxygen may be involved. The reaction takes place in three stages. A very large excess of the haloaldehyde is reacted with the acid salt of the hydroxy amine, suitably in a polar inert anhydrous organic solvent. There is thus formed a five membered oxazolidine ring (or a six-membered 1,3-tetrahydrooxazine ring) by condensation of the aldehyde group with the hydroxyl and the amine groups. This product is treated with an organic base,

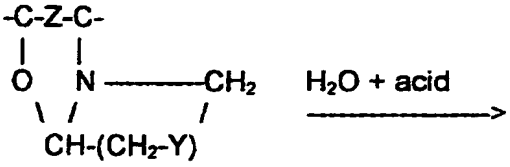
suitably a tertiary amine, whereby the elements of hydro-halic acid are eliminated between the halo moiety of the former halo aldehyde and the secondary amino group of the oxazolidine or 1,3-tetrahydrooxazine ring to form a fused ring structure by the addition of a 5 or 6 membered ring. The base is then neutralized with a weak acid suitably an organic acid such as glacial acetic acid. Treatment with aqueous acid, suitably an organic acid opens the oxazolidine or 1,3-tetrahydrooxazine portion of the fused ring. It will be understood by those skilled in the art that depending on the starting aldehyde, the final nitrogen containg ring may contain at least one additional hetero atom as mentioned above. The general reaction may be illustrated as follows:



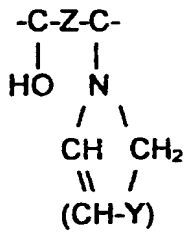
(III)



(IV)



(V)



(VI)

Wherein X' is halo, suitably bromo or iodo, preferably iodo,
Y is CH_2, OCH_2, CH_2-CH_2,
Z is nil or CH_2

When Z is nil, the aldehyde moiety forms a 5-membered oxazolidine ring as the first step of the reaction. When Z is CH_2, the aldehyde moiety forms a 6-membered 1,3-tetrahydrooxazine ring. While such ring formations are well known, in combination with the ring closure effected by the haloalkane side chain in a basic medium such as a tertiary amine in an anhydrous medium, the reaction is new and surprising.

Brief Description of the Drawings

FIGURE 1 is plot of volume changes of estrogen independent MXT mouse mammary cancers for different dosage levels of compounds of the present invention and DOX.

FIGURE 2 is plot of volume changes of estrogen independent MXT mouse mammary cancers for different dosage levels of a certain compound of the present invention, a prior art compound, DOX and a control.

FIGURE 3 is plot of the effect of certain cytotoxic LHRH analogs on the survival of mice with estrogen independent MXT mouse mammary cancers.

FIGURE 4 is a plot of tumor volume in male Copenhagen rats bearing rat Dunning R-3327-H prostate carcinoma transplants during the treatment with a prior art agonist and a certain compound of the present invention.

FIGURE 5 is a plot showing the effect of treatment with a certain compound of the present invention and the corresponding cytotoxic LH-RH analog on the tumor volume in rats with Dunning R-3327-H prostate cancer.

FIGURE 6 is a plot showing the effect of treatment with with a certain compound of the present invention and the corresponding cytotoxic LH-RH analog on the body weight of Copenhagen rats bearing Dunning R-3327-H prostate cancer.

FIGURE 7 is a plot showing inhibition of tumor growth achieved by treatment with a certain compound of the present invention and DOX.

Description of the Preferred Embodiments

The moiety Q, when substituted at R' by certain preferred groups, has submoiety designations of Q_1 through Q_8, of which Q_2 through Q_8 are novel cytotoxic moieties.

R' has the preferred values, leading to the desired Q_x moieties listed in parentheses as follows: NH_2 (Q_1), pyrrolidine-1-yl (Q_2), isoindoline-2-yl (Q_3), 3-pyrroline-1-yl (Q_4), 3-pyrrolidone-1-yl (Q_5), 2-pyrroline-1-yl (Q_6), 3-piperidone-1-yl (Q_7), or 1,3-tetrahydropyridine-1-yl(Q_8).

Thus if R-P is H and -R' is -NH_2, Q_1 is DOX, if R-P is H and -R' is pyrrolidine-1-yl, Q_2 is 3'-deamino-3'-(pyrrolidine-1"-yl)-doxorubicin (Q_2); if R-P is H and -R' is isoindoline-2-yl, Q_3 is 3'-deamino-3'-(isoindoline-2"-yl)-doxorubicin (Q_3); if R-P is H and -R' is 3-pyrroline-1-yl, Q_4 is 3'-deamino-3'-(3"-pyrroline-1"-yl)-

doxorubicin (Q_4); if R-P is H and -R' is 3-pyrrolidone-1-yl, Q_5 is 3'-deamino-3'-(3"-pyrrolidone-1"-yl)-doxorubicin (Q_5); if R-P is H and -R' is 2-pyrroline-1-yl, Q_6 is 3'-deamino-3'-(2"-pyrroline-1"-yl)-doxorubicin (Q_6); if R-P is H and -R' is 3-piperidone-1-yl, Q_7 is 3'-deamino-3'-(3"-piperidone-1"-yl)-doxorubicin (Q_7); if R-P is H and -R' is 1,3-tetrahydro-pyridine-1-yl, Q_8 is 3'-deamino-3'-(1",3"-tetrahydropyridine-1"-yl)-doxorubicin (Q_8).

The compounds incorporating the daunosamine nitrogen in a five membered ring with alkylating function are 10-50 times more active in vitro than their homolog counterparts, incorporating the daunosamine nitrogen in a six membered ring. (Such pairs are Q_5 and Q_7 as well as Q_6 and Q_8.)

In the preferred embodiments of the present invention, in the substance of formula Q^{14}-O-R-P, R and P are other than hydrogen. Where P is other than hydrogen, that is where it is P_1, P_2 and P_3, suitably where P_1 is an LH-RH agonist carrier, an LH-RH antagonist carrier or a shortened LH-RH analog carrier, P_2 is a shortened somatostatin analog and P_3 is a bombesin antagonist.

Suitably, P_1 is Aaa-Bbb-Ccc-Ser-Tyr-D-Lys(Xxx)-Leu-Arg-Pro-Ddd, wherein (Xxx) is hydrogen or a diamino substituent such as A_2Bu or A_2Pr wherein where:
Aaa is Glp, then Bbb is His, Ccc is Trp, and Ddd is Gly-NH_2,
Aaa is Ac-D-Nal(2), Ac-D-Phe or AcD-Phe(4Cl), then Bbb is D-Phe(4Cl) or D-Phe, Ccc is D-Pal(3) and D-Trp and Ddd is D-Ala-NH_2; and where Aaa-Bbb-Ccc is Ac, then Ddd is -NH-CH_2-CH_3;

P_2 is Aaa-Cys-Bbb-D-Trp-Lys-Ccc-Cys-Ddd-NH_2
wherein:
where Aaa is D-Phe, then Bbb is Tyr, Ccc is Val and Ddd is Thr or Trp; and

where Aaa is D-Trp, then Bbb is Phe, and Ccc and Ddd are Thr; and

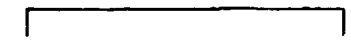
P_3 is Aaa-Gln-Trp-Ala-Val-Gly-His-Leu Bbb-NH_2
wherein: Aaa is nil, D-Tpi or D-Phe and Bbb is (CH_2-NH)Leu, (CH_2-NH)Phe, (CH_2-NH)Trp, (CH_2-N)Tac or (CH_2-N)DMTac.

In the novel compounds of the present invention incorporating analogs of LH-RH, the cytotoxic radical Q is attached to the D-Lys side chain on the LH-RH analogs or the (Xxx) group attached thereto, through a dicarboxylic acid spacer as formulated in Formula VII:

Aaa-Bbb-Ccc-Ser-Tyr-D-Lys(Xxx)$_m$(Q^{14}-O-R)$_n$-Leu-Arg-Pro-Ddd (VII)

where m is 1 or 0 and n is 1 or 2 provided that when m is 1 i.e. (Xxx) is A_2Bu or A_2Pr, n is 1 or 2, when m is 0 i.e. (Xxx) is H, n is 1.

In the novel compounds of the present invention incorporating analogs of somatostatin the cytotoxic radical Q is attached to the amino terminal of the somatostatin analogs through a dicarboxylic acid spacer as formulated in Formula VIII:



Q^{14}-O-R-Aaa-Cys-Bbb-D-Trp-Lys-Ccc-Cys-Ddd-NH_2 (VIII)

In the novel compounds of the present invention incorporating analogs of bombesin antagonists, the cytotoxic radical Q is linked to the amino terminal of the bombesin antagonists as formulated in Formula IX:

Q^{14}-O-R-Aaa-Gln-Trp-Ala-Val-Gly-His-Leu Bbb-NH_2 (IX)

Especially preferred embodiments of this invention are those peptide conjugates that contain Q_1 and Q_6 as the cytotoxic radicals and glutaric acid (n=3) as the dicarboxylic acid spacer forming a 14-O-ester bond with Q_1

(doxorubicin) or Q_6 (2-pyrrolino-doxorubicin) and a carboxamide bond with the peptide carrier.

The most preferred embodiments of this invention are cytotoxic LH-RH analogs of the following formulae:

1. Glp-His-Trp-Ser-Tyr-D-Lys(Q_1^{14}-O-glt)-Leu-Arg-Pro-Gly-NH_2;
2. Glp-His-Trp-Ser-Tyr-D-Lys(Q_6^{14}-O-glt)-Leu-Arg-Pro-Gly-NH_2;

cytotoxic somatostatin analogs of the following formulae:

3. Q_1^{14}-O-glt-D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2;

4. Q_6^{14}-O-glt-D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2;

5. Q_1^{14}-O-glt-D-Trp-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-NH_2;

6. Q_6^{14}-O-glt-D-Trp-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-NH_2;

7. Q_1^{14}-O-glt-D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Trp-NH_2; and

8. Q_6^{14}-O-glt-D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Trp-NH_2;

and cytotoxic bombesin antagonist analogs of the following formulae:

9. Q_1^{14}-O-glt-Gln-Trp-Ala-Val-Gly-His-Leu (CH_2-NH)Leu-NH_2;
10. Q_6^{14}-O-glt-Gln-Trp-Ala-Val-Gly-His-Leu (CH_2-NH)Leu-NH_2
11. Q_1^{14}-O-glt-D-Tpi-Gln-Trp-Ala-Val-Gly-His-Leu (CH_2-NH)Leu-NH_2; and
12. Q_6^{14}-O-glt-D-Tpi-Gln-Trp-Ala-Val-Gly-His-Leu (CH_2-NH)Leu-NH_2.

In the novel process of forming a partially saturated heterocyclic ring with the nitrogen of a vicinal or disjunct i.e., α,β- or α,γ-hydroxy amine the first step of the reaction is carried out in an anhydrous inert organic polar non-hydroxylic (aprotic) solvent, suitably dimethyl formamide using substantial excess, suitably a 30 fold excess of the halo aldehyde, 4-iodobutyraldehyde and 5-iodovaleraldehyde are especially effective. The invention is not limited to these however, bromo may be used in place of iodo. This reaction as well as the subsequent steps may be carried out at ambient temperature.

The basification step is carried out with an excess, suitably a 2-4 fold excess of an organic base. Tertiary amines such as trialkylamines are suitable for this purpose.

The thus formed bicyclic ring is opened to release the vicinal or disjunct hydroxyl group by treatment with an organic acid in the presence of water. Dilute aqueous trifuoracetic acid, suitably in an inert organic solvent such as acetonitrile may be used. The product is purified by removal ofthe volatiles under reduced pressure, excess halo compound extracted with hexane, and the residue purified on HPLC.

Abbreviations

For the description of the peptides and their derivatives of this invention, the conventional abbreviations for the amino acids are used as generally accepted in the peptide chemistry art and as recommended by the IUPAC-

IUB Commission on Biochemical Nomenclature (European J. Biochem., 138, 9-37 (1984).

The abbreviations for the individual amino acid residues are based on the trivial name of the amino acid, e.g. Glp is pyroglutamic acid, His is histidine, Trp is tryptophan, etc. The abbreviations indicate the L isomeric form of the amino acids, unless expressed otherwise, e. g., Ser is L-serine, and D-Lys is D-lysine.

Abbreviations of the uncommon amino acids in this invention are as follows: D-Nal(2) is D-3-(2-naphthyl)alanine, and D-Pal(3) is D-3-(3-pyridyl)alanine, D-Phe(4Cl) is D-4-chlorophenylalanine.

Peptide sequences are written according to the convention whereby the N-terminal amino acid is on the left and the C-terminal amino acid is on the right, e.g., Glp-His-Trp.

The formula, Leu (CH_2-NH)Leu-NH_2 describes a reduced peptide bond between a leucine and leucine amide residue at the C-terminal of a peptide sequence.

Other abbreviations used are:
A_2Bu: diaminobutyric acid
A_2Pr: diaminopropionic acid
BN: bombesin
BOP reagent: benzotriazole-1-yloxitris(dimethylamino)phosphonium hexa-fluorophosphate
DIPEA: N,N-diisopropylethylamine
DM-DOX: daunosamine modified doxorubicin
DMF: N,N-dimethylformamide
DMTac: 5,5-dimethyl-thiazolidine-4-carboxylic acid

DOX: doxorubicin

Fmoc: 9-fluorenylmethyloxycarbonyl

glt : -C(O)-CH_2-CH_2-CH_2-C(O)-, glutaryl

Glt_2O: glutaric anhydride

HOBt : 1-hydroxibenzotriazole

HO-glt-OH: glutaric acid

HOSu : N-hydroxysuccinimide

HPLC: high performance liquid chromatography

TFA: trifluoroacetic acid

Tac: thiazolidine-4-carboxylic acid

Tpi: 2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-3-carboxylic acid

A Beckman analytical HPLC system equipped with model 168 diode array detector and System Gold chromatography software (Beckman) was used to monitor the chemical reactions and to check the purity of the compounds of this invention. The column used was Dynamax C-18 (250x4.6 mm; pore size: 300Å; particle size:12 µm. The solvent system consisted of two components: (i) 0.1% TFA in water, and (ii) 0.1% TFA in 70% aqueous acetonitrile and used in linear gradient mode, growing 1% (ii) in 1 min., for monitoring the chemical reactions. The system was used in isocratic mode for purity control.

A Beckman model 342 semipreparative HPLC system was used for isolation and purification of the compounds of this invention. The column was Aquapore Octyl (250x10mm; pore size: 300Å; particle size: 15 µm). The solvent system was the same described for the analytical HPLC above.

Analysis

Bruker ARX300 NMR spectrometer (300MHz 1H frequency, 75MHz 13C frequency) and electrospray mass spectrometer Finnigan-MAT TSQ 7000 were used for the structure identification of the doxorubicin derivatives.

Synthesis of peptide carriers

The peptides of the invention are often administered in the form of pharmaceutically acceptable, nontoxic salts, such as acid additional salts. Illustrative of such acid addition salts are hydrochloride, hydrobromide, sulphate, phosphate, fumarate, glyconate, tannate, maleate, acetate, trifluoroacetate, citrate, benzoate, succinate, alginate, pamoate, malate, ascorbate, tartrate, and the like. If the active ingredient is to be administered in tablet form, the tablet may contain a pharmaceutically acceptable diluent which includes a binder, such as tragacanth, corn starch or gelatin, a disintegrating agent, such as alginic acid and a lubricant, such as magnesium stearate.

If administration in liquid form is desired, sweetening and/or flavoring may be used as part of the pharmaceutically-acceptable diluent, an intravenous administration in isotonic saline, phosphate buffer solutions or the like may be effected.

The pharmaceutical compositions will usually contain the peptide in conjunction with a conventional, pharmaceutically-acceptable carrier. Usually, the dosage will be from about 1 to about 100 micrograms of the peptide per kilogram of the body weight of the host when given intravenously; oral dosages will be much higher. Overall, treatment of subjects with these peptides is generally carried out in the same manner as the clinical treatment using other analogs of LHRH, somatostatin and analogs of doxorubicin.

These peptides can be administered to mammals intravenously, subcutaneously, intramuscularly, orally, intranasally or intravaginally to achieve biological hormonal effects through binding to specific receptors. In the case of LHRH analogs, these effects may include reversible suppression

of gonadal activity, and in the case of somatostatin analogs, inhibition of gastrointentinal function. Effective dosages will vary with the form of administration and the particular species of mammal being treated. An example of one typical dosage form is a physiological saline solution containing the peptide which solution is administered to provide a dose in the range of about 0.1 to 2.5 mg/kg of body weight. Oral administration of the peptide may be given in either solid form or liquid form.

The synthesis of the peptide carriers of the present invention can be performed by any techniques that are known to those skilled in the art of peptide chemistry. A summary of the suitable techniques can be found in M. Bodanszky, Principles of Peptide Synthesis, Springer-Verlag, Heidelberg, 1984. Techniques for solid phase peptide synthesis can be found in the textbook of J.M. Stewart and J.D. Young, Solid Phase Peptide Synthesis, Pierce Chem. Co., Rockford, IL, 1984 (2nd ed.) and in the review of G. Barany et al., Int. J. Peptide and Protein Res. 30, 705-739 (1987).

The synthesis of the LH-RH analog carriers used in this invention is detailed in the examples of US Patent 5,258,492, Sandor Bajusz and Andrew V. Schally, November 2, 1993 and in the articles of Bajusz et al., Proc. Natl. Acad. Sci. USA 85, 1637-1641 (1988) and 86, 6318-6322 (1989) and Janaky et al., Proc. Natl. Acad. Sci. USA, 89, 1023-1027 and 972-976 (1992).

The synthesis of the somatostatin analog carriers used in this invention is detailed in the examples of U.S. Patent 4,650,787, March 17, 1987, Andrew V. Schally and Ren Z. Cai. A description of the synthesis can also be found in the articles by Cai et al., Proc. Natl. Acad. Sci. USA 83, 1896-1900 (1986) and Proc. Natl. Acad. Sci. USA 84, 2502-2506 (1987).

The synthesis of the bombesin antagonist carriers used in this invention is detailed in the articles by Coy et al., J. Biol. Chem. 263, 5056-5060 (1988)

and 264, 14691-14697 (1989) and by Cai et al., Peptides 13, 267-271 (1992) and Proc. Natl. Acad. Sci. USA 91, 12664-12668 (1994).

The synthesis of the doxorubicin derivatives used in this invention and the formation of their conjugates with different peptide carriers is detailed in the following examples which are intended to be illustrative and not limiting:

EXAMPLE 1

Preparation and isolation of N-Fmoc-DOX^{14}-O-hemiglutarate

DOX HCl salt, 50 mg (86 µmol), was dissolved in 1 mL DMF and 30 mg(90 µmol) Fmoc-OSu was added followed by the addition of 31 µL (180 µmol) DIPEA. After stirring for three hours, the reaction was complete as assessed by analytical HPLC. The solvent was evaporated to dryness in Speed Vac high vacuum evaporator and the residue was crystallized by rubbing with 0.1% TFA in H2O. The crystals were filtered and washed once by cold ether to remove traces of excess Fmoc-OSu. After drying in a desiccator, m=62mg, of 98% pure N-Fmoc-DOX was obtained. Yield:94%

This intermediate was reacted overnight with 11.4 mg(100 µmol) Glt_2O in 1 mL anhydrous DMF in the presence of 26.1 µL (150 µmol) DIPEA. The solvent was evaporated in Speed Vac and the residual oil was solidified by rubbing with 0.1% aqueous TFA (v/v). The crude material thus obtained contains 70% N-Fmoc-DOX14-O-hemiglutarate, 20% unreacted N-Fmoc-DOX and 10% other impurities as assessed by analytical HPLC. This crude product can be used for the preparation of peptide DOX conjugates without further purification. When this crude material was dissolved in 20 mL 60% aqueous acetonitrile containing 0.1% TFA and applied on semipreparative HPLC, 45.7 mg, of 98% pure N-Fmoc-DOX14-O-hemiglutarate end product was obtained. (Yield: 64%.)

EXAMPLE 2

Preparation and isolation of 3'-deamino-3'-(pyrrolidine-1''-yl)-doxorubicin TFA salt (Q_2) and its 14-O-hemiglutarate (AN-193) TFA salt

DOX HCl salt, 50 mg(86 µmol), was dissolved in 1 mL DMF and 171 µL (1.3 mmol) 15 fold excess 1,4-diiodobutane was added followed by the addition of 45 µL (260 µmol) 3 fold excess DIPEA. The reaction mixture was stirred overnight at room temperature. After 16 hours the reaction was complete as assessed by analytical HPLC. The solvent is evaporated in Speed Vac and the residual oil is dissolved in 3 mL 0.1% TFA in H2O and extracted with ether to remove excess 1,4-diiodobutane. The aqueous extract was then applied on HPLC and m:41.6 mg, of 98% pure DOX derivative was obtained. (Yield 68%)

The 41.6 mg (58 µmol) 3'-deamino-3'-(pyrrolidine-1''-yl)-doxorubicin TFA salt (Q_2) thus obtained was reacted with 1.2 equivalent Glt_2O in dry DMF exactly as described in Example 1. The yield was 35% (16.9 mg) and the purity was 98%.

EXAMPLE 3

Preparation and isolation of 3'-deamino-3'-(isoindoline-2''-yl)doxorubicin TFA salt (Q_3)

DOX HCl salt, 50 mg(86 µmol), was dissolved in 1 mL DMF and 226 mg (1.3 mmol) 15 fold excess α,α'-dichloro-ortho-xylene was added followed by the addition of 45 µL (260 µmol) 3 fold excess DIPEA and catalytical amount of NaI. After 16 hours the solvents were removed with Speed Vac and the residue was dissolved in 3 mL 0.1% aqueous TFA and extracted with 3 mL

ether to remove the excess of the halogen compound. The crude material thus obtained was applied on HPLC. After purification 36 mg, 98% pure end product was obtained. (Yield: 55%)

EXAMPLE 4

Preparation and isolation of 3'-deamino-3'-(3"-pyrroline-1"-yl)-doxorubicin TFA salt (Q_4)

DOX HCl salt, 50 mg(86 µmol), was dissolved in 1 mL DMF and 136.8 µL (1.3 mmol) 15 fold excess cis-1,4-dichloro-2-butene (Aldrich) was added followed by the addition of 45 µL (260 µmol) 3 fold excess DIPEA. After 16 hours the solvents were removed in Speed Vac and the residue was dissolved in 3 mL 0.1% aqueous TFA and extracted with 3 mL hexane to remove the excess of the halogen compound. The crude material thus obtained was applied on HPLC. After purification 22.6 mg, 98% pure end product was obtained. (Yield:37%)

EXAMPLE 5

Preparation and isolation of 1-chloro-4-bromo-2-butanone (C_4H_6ClBrO) and 1-chloro-5-bromo-2-pentanone (C_5H_8ClBrO)

3-Bromopropionyl chloride, 100.8 µL (1 mmol), (Aldrich) was reacted with excess diazomethane in ether. After 1 hr the ethereal solution was eluted and spot tested on TLC. Thin layer chromatography aluminum sheets pre-coated with silica gel 60 F254 by Merck Art No. 5554 was used as the stationary phase and $CHCl_3$:MeOH 95:5 (v/v) as the mobile phase. For the spot test 2,4-dinitrophenylhydrazine reagent (Vogel: A textbook of Practical Organic Chemistry, page 1061, Third Edition, Longmans, New York.) was sprayed on the TLC sheet after elution. The diazomethylketone derivative

thus formed showed a yellow spot with Rf:0.3. The ethereal solution was then reacted with anhydrous HCl in ether converting the diazomethylketone to the desired end product, 1-chloro-4-bromo-2-butanone. This product showed a yellow spot, characteristic of oxo compounds, with Rf:0.8 in the same solvent system and with the spot test reagent described above. After evaporation of the solvent, the crude product was applied on a column (15 cm long, 2.5 cm in diameter) packed with 15 g silica gel, Merck, grade 9385, 230-400 mesh, pore size 60Å. The liquid, mobile phase was neat CHCl3. Fractions containing the desired end product (characterized by the spot test detailed above) were mixed and evaporated to dryness. M=1.5 g, clear oil was obtained. Yield: 80%.

1-chloro-5-bromo-2-pentanone was prepared from 4-bromobutyryl chloride exactly the same way as described for 1-chloro-4-bromo-2-pentanone, except that 4-bromobutyryl chloride was used instead of 3-bromopropionyl chloride. 1.6 g. clear oil was obtained. Yield: 80%.

EXAMPLE 6

Preparation and isolation of 3'-deamino-3'-(3"-pyrrolidone-1"-yl)-doxorubicin TFA salt (Q_5)

DOX HCl salt, 50 mg(86 µmol), was dissolved in 1 mL DMF and 241 mg (1.3 mmol) 15 fold excess 1-chloro-4-bromo-2-butanone was added followed by the addition of 45 µL (260 µmol) 3-fold excess DIPEA. After 16 hours the solvents were removed in a Speed Vac and the residue was dissolved in 3 mL 0.1% aqueous TFA and extracted with 3 mL hexane to remove the excess halogen compound. The crude material thus obtained was applied on HPLC. After purification, 20.6 mg, 98% pure end product was obtained. (Yield:33%)

EXAMPLE 7

Preparation and isolation of 3'-deamino-3'-(3"-piperidone-1"-yl)-doxorubicin TFA salt (Q_7)

DOX HCl salt, 50 mg(86 µmol), was dissolved in 1 mL DMF and 260 mg (1.3 mmol) 15 fold excess 1-chloro-5-bromo-2-pentanone was added followed by the addition of 45 µL (260 µmol) 3 fold excess DIPEA. After 16 hours the solvents were removed in a Speed Vac and the residue was dissolved in 3 mL 0.1% aqueous TFA and extracted with 3 mL hexane to remove the excess of the halogen compound. The crude material thus obtained was applied on HPLC. After purification, 18 mg, 95% pure end product was obtained. (Yield:28%)

EXAMPLE 8

Preparation and isolation of 4-iodobutyraldehyde and 5-iodovaleraldehyde

2-(3-Chloropropyl)-1,3-dioxolane (4-chloro-n-butyraldehyde ethylene acetal), 1.3 mL (10 mmol), (Fluka) was dissolved in 200 mL acetone containing 30 g (200 mmol, 20-fold excess) NaI. The solution was refluxed for 24 hours followed by evaporation to dryness. 100 mL ether was used to extract the organic material from the inorganic solid residue. The ethereal solution was then washed with 50 mL H2O, 50 mL 5% aqueous $Na_2S_2O_3$ solution and 3 times with 50 mL H_2O. The ether was removed in vacuo and the remaining oil was dissolved in 3 mL 50% aqueous acetic acid. After 1 hr 100 mL ether was added to this solution and the acetic acid as well as the ethylene glycol was removed by washing with 50 mL H_2O 3 times. The main product was eluted at Rf: 0.8 on TLC in neat $CHCl_3$. The spot test used for the aldehyde function was the same described for the ketones in Example 5. The ether was then removed and the black oil was applied on a column (15 cm long, 2.5 cm in

diameter) packed with 15 g silica gel, Merck, grade 9385, 230-400 mesh, pore size 60Å. The liquid, mobile phase was $CHCl_3$. Fractions containing the desired end product (characterized by the spot test detailed above) were mixed and evaporated to dryness. 1.6 g yellow oil was obtained. Yield: 80%.

5-Iodovaleraldehyde was obtained exactly the same way starting from 2-(4-chlorobutyl)-1,3-dioxolane (5-chloro-n-valeraldehyde ethylene acetal) (Fluka). 1.65 g yellow oil was obtained. Yield: 80%.

EXAMPLE 9

Preparation and isolation of 3'-deamino-3'-(2"-pyrroline-1"-yl)-doxorubicin TFA salt (Q_6)

DOX HCl salt, 50 mg(86 µmol), was dissolved in 1 mL DMF and 515 mg (2.6 mmol) 30-fold excess 4-iodobutyraldehyde was added followed by the addition of 45 µL (260 µmol, 3-fold excess) DIPEA. After 1 hour 100 µL glacial acetic acid was added to the reaction mixture which was then added dropwise to 5 mL of 0.1% TFA in 70% aqueous acetonitrile (solvent ii of the HPLC system). This solution was diluted with 2 mL 0.1% aqueous TFA solution followed by the removal of the acetonitrile in a Speed Vac. The resulting solution was extracted with hexane to remove the excess of the halogen compound. The material thus obtained was applied on HPLC. After purification 52 mg, 98% pure end product was obtained. (Yield:85%)

EXAMPLE 10

Preparation and isolation of 3'-deamino-3'-(1",3"-tetrahydropyridine-1"-yl)-doxorubicin TFA salt (Q_8)

DOX HCl salt, 50 mg(86 µmol), was dissolved in 1mL DMF and 552 mg (2.6 mmol) 30-fold excess 5-iodovaleraldehyde was added followed by the addition of 45 µL (260 µmol) 3-fold excess DIPEA. After 1 hour 100 µL glacial acetic acid was added to the reaction mixture which was then added dropwise to 5 mL of 0.1% TFA in 70% aqueous acetonitrile (solvent ii of the HPLC system). This solution was diluted with 2 mL 0.1% aqueous TFA solution followed by the removal of the acetonitrile in a Speed Vac. The resulting solution was extracted with hexane to remove the excess halogen compound. The material thus obtained was applied on HPLC. After purification, 46 mg, 98% pure end product was obtained. (Yield:75%)

EXAMPLE 11

Preparation and isolation of cytotoxic LH-RH agonist analog containing DOX. ([D-Lys^6(DOX^{14}-O-glt)]LH-RH, $Q_1{}^{14}$gL)

[D-Lys^6]LH-RH, 60 mg (37.5 µmol), and 52 mg (64% pure, 37.5 µmol) N-Fmoc-DOX14-O-hemiglutarate, (see Example 1), was dissolved in 1mL DMF and 22 mg (50 µmol) BOP reagent (Aldrich), 13.5 mg (100 µmol) HOBt as well as 52 µL (300 µmol) DIPEA was added. After stirring for 1 hr at room temperature the reaction is complete. The solvents were evaporated and the residual oil was crystallized by 3 mL ethyl acetate and then washed twice with 3 mL ethyl acetate. The 90 mg crude solid material was then dissolved in 3 mL DMF and 300 µL piperidine was added. After 5 minutes, the reaction was placed into an ice bath and was acidified by the addition of a mixture of 300 µL TFA, 700 µL pyridine and 2 mL DMF. After evaporation of the solvents, the residual oil was solidified by ethyl acetate. The crude solid thus obtained, was dissolved in 1 mL 70% aqueous acetonitrile containing 0.1% TFA (i) and diluted with 3 mL 0.1% aqueous TFA (ii) and applied on semipreparative HPLC. 40 mg (14.8 µmol) 98% pure end product was obtained. Yield: 48%.

EXAMPLE 12

Preparation of cytotoxic LH-RH agonist analog containing 2-pyrrolino-DOX ([D-Lys^6(2-pyrrolino-DOX^{14}-O-glt)]LH-RH, $Q_6{}^{14}$gL)

$Q_1{}^{14}$gL, 11.2 mg (5 µmol), (see Example 11) was dissolved in 200 µL DMF and 30 mg (150 µmol, 30-fold excess) 4-iodobutyraldehyde (Example 8) was added followed by the addition of 3 µL (17 µmol) DIPEA. After 1 hour, the reaction was complete (see Example 9) and 10 µL glacial acetic acid was added to the reaction mixture which was then added dropwise to 1 mL 0.1% TFA in 70% aqueous acetonitrile. This solution was then diluted with 1 mL 0.1% aqueous TFA and the acetonitrile was removed in vacuo. The remaining aqueous solution was then extracted with 1 mL hexane and applied on HPLC. m:7.6 mg, 99% pure end product was obtained. (Yield: 66%.)

EXAMPLE 13

Preparation and isolation of a cytotoxic somatostatin analog containing DOX

(DOX^{14}-O-glt-D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2, $Q_1{}^{14}$gS)

D-Phe-Cys-Tyr-D-Trp-Lys(Fmoc)-Val-Cys-Thr-NH_2, 20 mg (14.5 µmol) (Proc. Natl. Acad. Sci. USA 1986, pp. 1986-1990) and 20 mg (64% pure, 14.5 µmol) N-Fmoc-DOX14-O-hemiglutarate (Example 1) was dissolved in 200 µL DMF and 8.8 mg (20µmol) BOP reagent (Aldrich), 5.4 mg (40 µmol) HOBt as well as 17 µL (100 µmol) DIPEA was added. After stirring for 1 hour at room temperature, the reaction was complete. After removal of the solvents in vacuo, the residue was crystallized by ethyl acetate. This solid material was

then dissolved in 1 mL DMF and 100 µL piperidine was added. After 7 min the reaction was placed into an ice bath and was acidified by the addition of a mixture of 100 µL TFA, 300 µL pyridine and 2 mL DMF. After evaporation of the solvents, the residual oil was solidified by ethyl acetate. The crude solid thus obtained was dissolved in 1 mL 70% aqueous acetonitrile containing 0.1% TFA (i) and diluted with 3 mL 0.1% aqueous TFA (ii) and applied on semipreparative HPLC. 9.7 mg (5.1 µmol) 95% pure end product was obtained. Yield: 35%.

EXAMPLE 14

Preparation of cytotoxic somatostatin analog containing 2-pyrrolino-DOX

(2-pyrrolino-DOX^{14}-O-glt-D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2, $Q_6{}^{14}$gS)

D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2 (6.4 mg, 5µmol) was dissolved in 100 µL DMF and 2-pyrrolino-DOX^{14}-O-hemiglutarate (4.1mg, 5µmol) was added, followed by BOP reagent (4.4 mg, 10µmol) HOBt (100µmol) and DIPEA (50µmol). After stirring for 2 hr at room temperature, the reaction mixture was acidified by 20µL AcOH and diluted with 500µL 70% aqueous acetonitrile containing 0.1% TFA and further diluted with 700µL 0.1% aqueous TFA and applied on HPLC. 3.9 mg (Yield:40%) of 99% pure end product was obtained.

2-Pyrrolino-DOX^{14}-O-hemiglutarate was prepared by reacting DOX14-O-hemiglutarate with 4-iodobutyraldehyde as described in EXAMPLE 9.

DOX^{14}-O-hemiglutarate was prepared from N-Fmoc-DOX^{14}-O-hemiglutarate by cleaving the Fmoc protecting group as described in EXAMPLE 11. (Yield: 40%)

EXAMPLE 15

Preparation and isolation of a cytotoxic bombesin antagonist containing DOX (DOX^{14}-O-glt-Gln-Trp-Ala-Val-Gly-His-Leu (CH_2-NH)Leu-NH_2, $Q_1{}^{14}$gB)

Gln-Trp-Ala-Val-Gly-His-Leu (CH_2-NH)Leu-NH_2, 20 mg (15.8 µmol) (Int. J. Peptide Protein Res. 38, 1991, pp. 593-600) and 22 mg (64% pure, 15.8 µmol) N-Fmoc-DOX14-O-hemiglutarate (Example 1) was dissolved in 200 µL DMF and 8.8 mg (20 µmol) BOP reagent (Aldrich), 5.4 mg (40 µmol) HOBt as well as 17 µL (100 µmol) DIPEA was added. After stirring for 1 hour at room temperature the reaction was complete. After removal of the solvents in vacuo, the residue was crystallized by ethyl acetate. This solid material was then dissolved in 1 mL DMF and 100 µL piperidine was added. After 5 min the reaction was placed into an ice bath and was acidified by the addition of a mixture of 100 µL TFA, 300 µL pyridine and 2 mL DMF. After evaporation of the solvents, the residual oil was solidified by ethyl acetate. The crude solid thus obtained was dissolved in 1 mL 70% aqueous acetonitrile containing 0.1% TFA (i) and diluted with 3 mL 0.1% aqueous TFA (ii) and applied on semipreparative HPLC. 13.5 mg (7.1 µmol) 98% pure end product was obtained. Yield: 45%.

EXAMPLE 16

Preparation and isolation of a cytotoxic bombesin antagonistic analog containing 2-pyrrolino-DOX

2-pyrrolino-DOX^{14}-O-glt-Gln-Trp-Ala-Val-Gly-His-Leu (CH_2-NH)Leu-NH_2, $Q_6{}^{14}$gB

Q_1^{14}gB, 9.5 mg (5 µmol), (Example 15) was dissolved in 200 µL DMF and 30 mg (150 µmol, 30-fold excess) 4-iodobutyraldehyde (Example 8) was added followed by the addition of 3 µL (17 µmol) DIPEA. After 1 hour the reaction was complete (Example 9) and 10 µL glacial acetic acid was added to the reaction mixture which was then added dropwise to 1 mL 0.1% TFA in 70% aqueous acetonitrile. This solution was then diluted with 1 mL 0.1% aqueous TFA and the acetonitrile was removed in vacuo. The remaining aqueous solution was then extracted with 1 mL hexane and applied on HPLC. 6 mg 98% pure end product was obtained. (Yield:60%.)

Determination of in vitro cytotoxic activity

MXT estrogen-independent mouse mammary carcinoma cell line was obtained from Dr. Gunter Berndhardt, University of Regensburg, Germany. All the other cell lines used in the determination of the antiproliferative activity of the compounds of this invention were obtained from the American Type Culture Collection (ATCC).

For the evaluation of the activity of the analogs, a colorimetric cytotoxicity assay in microtitration plates was used based on quantification of biomass by staining cells with crystal violet, which correlates very well with determination of cell numbers. (Reile et al.; Anal. Biochem. 187, 262-267, 1990; Bernhardt G. et al, J. Cancer Res. Clin. Oncol. (1992), 118, 35-43; Spruss Th. et al, J. Cancer Res. Clin. Oncol 117, 435-443, 1991; Gillies, R. J., Anal. Biochem. 159, 109-113, 1986; Kueng, W. et al.; Anal. Biochem., 182 16-19, 1989.)

Assay Protocol

One to two days after seeding cells in 96-well plates the culture medium is exchanged with fresh medium containing the compounds to be tested and

fresh medium only for the control cultures. After varying time of incubation, cells are fixed with glutaric dialdehyde and stored under fetal bovine serum (FBS) at 4oC until the end of the experiment. Cells are stained with crystal violet and bound stain is extracted with 70% aqueous EtOH. Optical density is measured with EIA Reader (Bio-Tek Instruments) or Biomek 1000 (Beckman) at 590 nm or 600 nm, respectively. Each data point represents the mean value of eight culture wells. T/C values are calculated as T/C= (T-C0)/(C-C0) where T= optical density of treated cultures, C= optical density of control (untreated) cultures, C0= optical density of cultures at the start of incubation (t=0).

EXAMPLE 17

In vitro cytotoxic activity of daunosamine modified derivatives of DOX

Table 17-1 demonstrates the effects of doxorubicin and its daunosamine modified derivatives on MCF-7 human mammary carcinoma cell line in vitro.

Cytotoxic radicals having their daunosamine N incorporated into a five-membered ring with a reactive function are 5 to 50 times more active than their homolog counterpart with a six-membered ring as the examples of 3-pyrrolidono-DOX (Q_5) and 3-piperidono-DOX (Q_7) as well as 2-pyrrolino-DOX (Q_6) and 1,3-tetrahydro-pyridino-DOX (Q_8).

Table 17-1: Effects of Doxorubicin and its Daunosamine modified derivatives on MCF-7 Human Mammary Carcinoma CelL Line in vitro

Compound	Incubation Time (hr.)	T/C Value at (M)					
		$3x10^{-10}$	10^{-9}	$3x10^{-9}$	10^{-8}	$3x10^{-8}$	10^{-7}
Doxorubicin (DOX)	70				98	82	54
	120				95	66	33
Pyrrolidino-DOX (Q_2)	70				97	25	-26
	120				94	17	-19
Piperidino-DOX (AN-183)	70			114	70	4	
	120			109	67	0	
Isoindolino-DOX (Q_3)	70				118	86	-11
	120				108	77	-29
3-Pyrrolino-DOX (Q_4)	70			106	72	-3	
	120			97	65	-5	
3-Pyrrolidono-DOX (Q_5)	70			87	30	-28	
	120			67	25	-10	
3-Piperidono-DOX (Q_7)	70			96	80	59	
	120			97	70	43	
2-Pyrrolino-DOX(Q_6)	70	50	-3	-18			
	120	26	2	-9			
1,3-Tetrahydro pyridino-DOX (Q_8)	70	96	88	69			
	120	99	93	62			

Cells were incubated in IMEM media containing 5% HI-DCC-FBS (heat inactivated dextran coated charcoal treated fetal bovine serum) on 96 well plates. Relative cell number in treated and control plates was determined by

the crystal violet staining method and was expressed as T/C values where $T/C=(T-C_0/C-C_0) \times 100$ [T= absorbance of treated cultures, C= absorbance of control cultures, C_0= absorbance of cultures at the start of incubation (t=0). The measured absorbance is proportionate to the cell number.]

Lower T/C values indicate a decrease in the survival of cancerous cells due to treatment. That is to say, 75 would indicate 75% survival of cells as compared to 100% for control or 25% inhibition.

EXAMPLE 18

Full retaining of in vitro cytotoxic activity of DOX in LH-RH agonist peptide conjugate $Q_1{}^{14}$gL and superactive 2-pyrrolino-DOX (Q_6) in LH-RH agonist peptide conjugate $Q_6{}^{14}$gL.

Table 18-1 demonstrates the effects doxorubicin and its daunosamine modified derivative, 2-pyrrolinodoxorubicin (Q_6) in comparison with their conjugates with LH-RH agonistic analog, [D-Lys^6]LH-RH ($Q_1{}^{14}$gL and $Q_6{}^{14}$gL, respectively) on the growth of MCF-7 human mammary carcinoma cell line and MXT estrogen independent mouse mammary carcinoma cell line in vitro.

Table 18-1:

Compound	Incu-bation Time (hr.)	T/C Value on MCF-7 Cell Line at Conc.(M)							
		$3x10^{-11}$	10^{-10}	$3x10^{-10}$	10^{-9}	$3x10^{-9}$	10^{-8}	$3x10^{-8}$	10^{-7}
Doxorubicin*	70						98	82	54
	120						95	66	33
$Q_1{}^{14}gL$	70						111	89	63
	120						78	55	28
Q_6	70		50		-3	-18			
	120		26		-2	-9			
$Q_6{}^{14}gL$	70		74		28	-24			
	120		60		16	-14			
Compound	Incu-bation Time (hr.)	T/C value on MXT cell line at Conc.(M)							
		$3x10^{-11}$	10^{-10}	$3x10^{-10}$	10^{-9}	$3x10^{-9}$	10^{-8}	$3x10^{-8}$	10^{-7}
Doxorubicin	26						85	90	59
	50						74	60	43
$Q_1{}^{14}gL$	26						87	91	73
	50						71	59	50
Q_6	28	90	78	56					
	69	52	6	-13					
$Q_6{}^{14}gL$	28	91	78	64					
	69	59	15	-11					

MCF-7 cells were incubated in IMEM media containing 5% HI-DCC-FBS on 96 well plates. MXT cells were incubated in RPMI 1640 media containing 0.6 g/L L-glutamine and 10% FBS.

*Determined as in Table 17-1.

EXAMPLE 19

Table 19-1 demonstrates that the in vitro cytotoxic activity of the somatostatin analogs containing DOX of the invention is fully retained.

Table 19-1: Effects of Cytotoxic Analogs of Somatostatin Containing Doxorubicin on the Growth of MIIA PaCa-2 Human Pancreatic Cancer Cell Line in Vitro

Compound	Incubation Time (hr.)	T/C Value at Concentration (M)		
		10^{-8}	10^{-7}	10^{-6}
DOX^{14}-O-glt-S-98* ($Q_1{}^{14}gS^{98}$)	28	93	95	32
	76	103	11	-3
Carrier Analog S-98*	28	-	-	96
	76	-	-	98
DOX^{14}-O-glt-S-121** ($Q_1{}^{14}gS^{121}$)	28	93	82	35
	76	97	10	-4
Carrier Analog S-121**	28	-	-	76
	76	-	-	96
Doxorubicin	28	95	64	-28
	76	71	10	-7

Cells were incubated in RPMI 1640 media containing 10% fetal bovine serum on 96 well plates.

*D-Trp-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-NH_2;

**D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2;

EXAMPLE 20

Effects of Cytotoxic Analogs of Bombesin Antagonists Containing Doxorubicin on the Growth of CFPAC-1 Human Pancreatic Cancer Cell In Vitro

Table 20-1 demonstrates that the in vitro cytotoxic activity of bombesin antagonistic analogs containing DOX of the invention is fully retained.

Table 20-1

Compound	Incubation Time (hr.)	T/C Value at Concentration (M)			
		$3x10^{-8}$	10^{-7}	$3x10^{-7}$	10^{-6}
DOX^{14}-O-glt	66	95	81	44	9
B-94	95	95	57	28	4
($Q_1{}^{14}$gB)	137	94	28	19	0
B-94*	66	99	106	104	100
	95	97	99	99	96
	137	98	98	100	96
DOX^{14}-O-glt-B-50	66	102	78	39	5
	95	97	55	24	-1
	137	92	28	19	-2
B-50**	66	100	93	99	93
	95	98	100	102	98
	137	97	98	99	98
DOX	66	88	52	15	-7
	95	73	32	10	-6
	137	49	20	7	-4

Cells were incubated in IMDM media containing 10% fetal bovine serum on 24 well plates.

* Gln-Trp-Ala-Val-Gly-His-Leu-ψ(CH_2-N)-Leu-NH_2

** D-Phe-Gln-Trp-Ala-Val-Gly-His-Leu-ψ(CH_2-N)-Tac-NH_2

Preserved Binding Properties of Hormone Derivatives

EXAMPLE 21

Hormonal activities and receptor binding potencies of cytotoxic LH-RH agonist analogs $Q_1{}^{14}$gL ([D-Lys^6]LH-RH carrying DOX) and $Q_6{}^{14}$gL ([D-Lys^6]LH-RH carrying 2-pyrrolino-DOX) in comparison with the carrier peptide, [D-Lys^6]LH-RH

Table 21-1

Compound	Hormonal activity* (LH-response rel. to LH-RH=1)	IC50** value for rat pituitary receptors (nM)	IC50** value for breast cancer receptors (nM)
$Q_1{}^{14}$gL	15	2.29	7.24
$Q_6{}^{14}$gL	10	5.59	6.70
[D-Lys^6]LH-RH	8	2.26	1.80

In Table 21-1

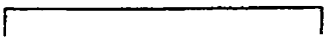
*LH responses to the analogs were determined in dispersed rat pituitary cell superfusion system as described in S. Vigh and A. V. Schally, Peptides 5, 241-247 (1984).

**Binding affinities of the analogs to rat pituitary LH-RH receptors and human breast cancer receptors were determined in competitive binding experiments using [125I] labeled [D-Trp6]LH-RH as radio ligand as described in B. Szoke et al., Peptides, 15(2), 359-366 (1994). The binding affinities were expressed by IC50 values, the concentration of unlabeled analog required to inhibit 50% of the specific binding of the radio ligand.

EXAMPLE 22

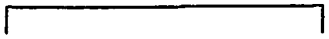
Somatostatin analogs inhibit the secretion of growth hormone (GH) from perfused rat pituitary as it is described by Carlson et al., Thyrotropin-releasing hormone stimulation and somatostatin inhibition of growth hormone secretion from perfused rat adenohypophyses Endocrinology, 94, 1709-(1974). Accordingly, this method was used to compare the cytotoxic somatostatin analogs of the present invention to their parent carrier molecules with respect to their hormonal activities.

Inhibition of human growth hormone-releasing hormone (hGH-RH(1-29)NH_2) induced growth hormone release from superfused rat pituitary cells by somatostatin analogs S-98-I



D-Trp-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-NH_2;

and S-121



D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2;
in comparison with their cytotoxic derivative, $Q_1{}^{14}gS^{98\text{-}1}$(DOX^{14}-O-glt-S-98-I) and $Q_1{}^{14}gS^{121}$ (DOX^{14}-O-glt-S-121), respectively.

In rat pituitary superfusion system, the somatostatin analogs were administered for 3 min at 1 nM dose simultaneously with 1 nM hGH-RH(1-29)NH_2. The infusion of the somatostatin analogs was maintained for another 6 min. GH responses to 3 min administration of 1 nM hGH-RH(1-29)NH_2 were determined during the perfusion of the somatostatin analogs (0 min) and 30, 60 and 90 min after the administration stopped. The data are presented in Table 22-1.

Table 22-1

Somatostatin analogs	GH release** induced by 3 min administration of 1 nM hGH-RH(1-29)NH2 at different time points after infusion of the somatostatin analogs			
	0 min	30 min	60 min	90 min
S-98-I	2.9	94.7	117.6	-
$Q_1{}^{14}gS^{98}$	0	90	89.7	-
S-121	7.8	62.2	57.3	77.9
$Q_1{}^{14}gS^{121}$	8.8	58.5	54.3	67.7

**Expressed as percentage of GH release induced by 3 min infusion of 1 nM hGH-RH (1-29)NH2 prior to the administration of the somatostatin analogs.

EXAMPLE 23

Receptor binding studies with cytotoxic bombesin antagonists

Radio iodination of [Tyr^4]BN (Sigma) using a Bio-Rad Enzymobead Radio Iodination kit and isolation of mono-iodinated [^{125}I-Tyr^4]BN was performed as described earlier (1). Binding of labeled [Tyr^4]BN and displacement by cytotoxic bombesin antagonist analog, $Q_6{}^{14}gB$ was conducted using confluent Swiss 3T3 cells (obtained from the American Type Culture Collection) in 24-well plates in a modification (2) of the method of Kris et al (3). Three to five days after seeding, the confluent cells were washed twice with Hanks' Balanced Salt Solution (HBSS) and incubated for 30 min at 37oC with 50 pM [^{125}I-Tyr^4]BN in the absence or presence of several concentrations of unlabeled competitors ($Q_6{}^{14}gB$ or BN) in a total volume of 0.5 ml binding buffer (DMEM with 50 mM HEPES, 0.1% bovine serum albumin (BSA), 5 mM MgCl2 and 100 µg/ml bacitracin, pH: 7.4). Nonspecific binding was

determined in the presence of 1 μM unlabeled ligand. After three washings with ice-cold HBSS containing 0.1% BSA (pH: 7.4) the cells were detached with 0.05% Trypsin/ 0.53 mM EDTA solution and transferred to tubes. Radioactivity was measured with a gamma-counter (Micromedic Systems Inc, Huntsville, AL). Binding data were evaluated using radio ligand binding analysis programs by McPherson (4). K_i values presented in Table 23-1, were calculated according to the formula of Cheng and Prusoff (5).

1. Halmos, et al., Cancer Letters, 85, 111-118 (1994)
2. Cai, et al., Proc. Natl. Acad. Sci., USA 91:12664 -12668, (1994.)
3. Kris, et al., J. Biol. Chem, 262: 11215-11220, (1987.)
4. McPherson, G.A., J.Pharmaco Methods, 14: 213-228, (1985)
5. Cheng and Prusoff, Biochem. Pharmacol. 22:3099-3108, (1973)

Table 23-1

Characterization of the specific binding of cytotoxic bombesin antagonist Q_6^{14}gB (2-pyrrolino-DOX^{14}-O-glt-Gln-Trp-Ala-Val-Gly-His-Leu-ψ-(CH_2-N)Leu-NH_2 to bombesin receptors on Swiss 3T3 cell line in comparison with bombesin

Compound	Ki (nM)
Bombesin	1.2
Q_1^{14}gB	1.0

Comparative Effectiveness and Toxicity of Hormone Conjugates vs. Cytotoxic Radical Alone

EXAMPLE 24

Treatment with 2-pyrrolino-DOX (Q_6), cytotoxic LH-RH agonist analog Q_6^{14}gL ([D-Lys^6]LH-RH linked to Q_6^{14}-O-hemiglutarate) and (DOX) on estrogen independent MXT mouse mammary cancers (KS-49)

In order to compare the tumor inhibitory activity of cytotoxic doxorubicin derivative, Q_6 and its targeted cytotoxic peptide conjugate, Q_6^{14}gL as well as the well known antineoplastic agent, DOX and to determine the optimal way of administration and the nontoxic doses, LH-RH receptor positive MXT (3.2) ovex tumor pieces (1 mm^3) were implanted s. c. in female B_6D_2F1 mice. One day after transplantation the mice were randomly divided into groups of five animals and the treatment started. The compounds were dissolved in 0.1 % trifluoroacetic acid (pH 2) and given intraperitoneally. Groups, treatment schedules and doses as well as average survival times are shown in Table 24-1. Results are summarized in Table 24-2 and Figure 1.

Table 24-2 shows the effect of treatment with Q_6 and cytotoxic LH-RH analog Q_6^{14}gL on tumor volumes and survival of mice with estrogen-independent breast cancers. As is shown in Table 24-2, 1.25 nmol Q_6 administered on day 1, 2, 7,8,14 and 15, (Group 2) exerted strong toxicity characterized with an average survival of 17.4 days, which is significantly shorter than that of the untreated control group. In comparison, the same dosage of Q_6^{14}gL (Group 6) resulted in an average survival of 30.8 days, which is significantly longer than that of the untreated control group. Higher efficacy of Q_6^{14}gL over Q_6 can also be demonstrated by comparing the average final tumor volumes in Group 2 (1065 mm^3 at day 16) and in Group 6 (863 mm^3 at day 31).

Similar conclusions can be demonstrated by comparing Q_6 and Q_6^{14}gL in a different treatment schedule where 0.5 nmol of the drugs were administered five days a week for three consecutive weeks.

Doxorubicin at a toxic dose (total amount: 1560 nmol, average survival: 20 days) could not eradicate the tumor, while treatment with $Q_6^{14}gL$ at nontoxic dose (total amount: 7 nmol, average survival: >31 days) led to the survival of 2 out of 5 animals, without development of the tumor.

Table 24-1

No of group	Admin.	Dose/ Inj. (nmol)	Dose/ Inj. (µg)	Inj. /week	Days between Injection	Weeks Admin.	Total Amt. Recd	Aver. surviv.* day
1	Control							22
2	Q_6	1.25	0.92	2	5			17.5
3		0.5	0.37				7.5	19.6
4		0.25 *	0.19	5	2		9.5	14.6
5		0.2	0.15				21	13.0
6	$Q_6^{14}gL$	1.25	2.9	2	5	3		30.8
7		0.5	1.16				7.5	26.8
8		0.25 *	0.58	5	2		9.5	18.4
9		0.2	0.46				21	13.6
10		3.5	8.12				7	>31
11		4	9.28	1	6	2	8	
12		5	11.6				10	13.4
13	DOX	520	340			3	1560	20.0

* From day 9 to day 12, dose was raised to 2.5 nmol From day 9 to day 12, dose was raised to 5.0 nmol

*Survival

Table 24-2

No	Group	Mode of administration					Final Tumor Volume (mm^3)	Day of Measurement	Average survival (days)	Number of surviving mice without tumor from 5 mice per group	
		Dose/ inj. (nmol)	No of injections per week	Pause between injections (days)	Duration of treatment (weeks)	Total amount injected (nmol)				on day 18	on day 31
1	Control						7322	21	22.0±1.6	0	0
2	Q_6	1.25	2	5	3	7.5	1065	16	17.4±0.2	0	0
6	$Q_6{}^{14}gL$	1.25	2	5	3	7.5	863	31	30.8±0.4 **	2	0
3	Q_6	0.5	5	2	3	7.5	2531	18	19.6±0.7	0	0
7	$Q_6{}^{14}gL$	0.5	5	2	3	7.5	3978	31	26.8±2.6 **	1	0
10	$Q_6{}^{14}gL$	3.5	1	6	2	7	669	31	>31 **	4	2
12	$Q_6{}^{14}gL$	5.0	1	6	2	10	0	10	13.4	0	0
13	DOX	520	1	6	3	1560	1560	28	20	1	0

** Survival is significantly longer ($p<0.01$) Survival is significantly shorter ($p<0.01$) or ($p<0.05$) as compared with control data using Duncan's test.

EXAMPLE 25

Effects of a single treatment with (DOX), cytotoxic LH-RH analogs T-107 and $Q_1{}^{14}$gL on estrogen independent MXT mouse mammary cancers (KS-55)

Test Compounds:

$Q_1{}^{14}$gL: Doxorubicin^{14}-O-hemiglutarate linked to [D-Lys^6]LH-RH

T-107: N-glutaryl-doxorubicin linked to [D-Lys^6]LH-RH Proc. Natl. Acad. Sci. Vol. 89. Pp. 972-976 (1992)); and

DOX

The assays were run as follows:

In order to determine the maximum tolerated doses and compare the effects, MXT (3.2) ovex tumor pieces (1 mm3) were implanted s.c. in female B_6D_2F1 mice. One day after transplantation the mice were randomly divided into groups of five animals and they were treated with a single injection i.p. The groups and doses are shown in the Table 25-1. The table also shows the numbers of mice that had tumors when volume was measured and the average survival times for groups. Tumor volume changes are shown in Figure 2. The compounds were dissolved in 0.1% TFA (pH: 2.0). Tumor volume was measured on days 10, 13, 17, and 20.

As shown in Table 25-1 and Figure 2, T-107, ([D-Lys^6]LH-RH linked to N-glutaryl-DOX) is completely ineffective in inhibiting the growth of this tumor at a dose of 850 nmol/20 g mouse. In contrast, $Q_1{}^{14}$gL, ([D-Lys^6]LH-RH linked to 14-O-glutaryl-DOX) exerted strong suppression of tumor growth (Figure) at a nontoxic dose of 650 nmol/20 g mouse. DOX alone was highly toxic (average survival time: 13.6 days) at a single dose of 650 nmol/20 g mouse and significantly less effective, than $Q_1{}^{14}$gL (Figure 2).

Table 25

No.	Group	Dose			Number of tumorous mice/number of surviving mice				Average survival
		nmol/ 20 g	µg/ 20 g	µmol/kg	Day 10	Day 13	Day 17	Day 20	days
1	Control				5/5	5/5	5/5	5/5	21.2±0.3
2	$Q_1^{14}gL$	680	1520	34	1/4	2/4	2/4	3/4	28.6±693 5.3±25**
3	$Q_1^{14}gL$	710	1587	35.5	2/4	3/4	3/4	3/4	26.0±663 2.0±34*
4	$Q_1^{14}gL$	760	1698	38	3/5	4/5	4/5	(Sacr.)	(Sacr.)
5	DOX	650	427	32.5	3/3	2/2	1/1	1/1	13.6±25
6	DOX	700	460	35	2/3	2/3	2/2		15.2±24
7	DOX	750	493	37.5	1/1				7.8±1.3
8	T-107	750	1676	37.5	5/5	5/5	5/5	4/4	21.8±05
9	T-107	850	1900	44.4	5/5	5/5	5/5	4/4	21.6±07

*Survival is significantly shorter ($p<0.01$) than that of controls

**Survival is significantly longer ($p<0.01$) or * ($p<0.05$) as compared with control (one mouse which died accidentally on day 2 was left out from these two groups.

EXAMPLE 26

Effect of cytotoxic LH-RH analogs on estrogen independent MXT mouse mammary cancers (KS-47)

Substances used for treatment

In an earlier experiment, Q_2 at 20 nmol daily dose for 17 days had only a moderate inhibitory effect on tumor growth, and it was toxic at 40 nmol dose (mean survival was 14.6 days). A daily dose of 30 nmol was chosen for the present experiment, which compared the efficacy and toxicity of $Q_2{}^{14}$gL (Q_2 coupled to [D-Lys^6]LH-RH), Q_2 (pyrrolidino-doxorubicin), [D-Lys^6]LH-RH, and [D-Lys^6]LH-RH + Q_2.

MXT (3.2) ovex tumor pieces (1 mm^3) were transplanted in female B_6D_2F1 mice. The treatment started one day after transplantation and was continued for 12 days by i.p. injections once a day. All groups received equimolar amounts of the compounds as shown in Table 26-1. Tumors were measured on days 10, 14 and 18, and tumor volume was calculated. The data are shown in Table 26-1 and in Figure 3.

Treatment with a daily dose of 30 nmol of daunosamine modified doxorubicin analog Q_2 (pyrrolidino-DOX) resulted in strong inhibitory effect on tumor growth (tumor volume: 144 mm^3 at day 14 vs. 1391 mm^3 for the control group), but exerted severe toxicity killing all the animals before the end of the experiment (mean survival 17.9 days). Similarly, Q_2 combined (mixture) with [D-Lys^6]LH-RH resulted in strong tumor inhibitory effect (tumor volume: 80 mm^3 at day 14) but the mean survival (18.5 days) was significantly shorter than that of the untreated control group (23.1 days). As a result of the treatment with $Q_2{}^{14}$gL, (Q_2 covalently linked to [D-Lys^6]LH-RH) two animals died, one at day 16 and another at day 26. From the 8 surviving animals only one developed tumors at the last measurement at day 18 and they all looked

healthy, but later on all of them started to develop the tumors. The mean survival for this group was significantly longer (28.3 days), than that of the control group. Treatment with [D-Lys^6]LH-RH alone did not affect tumor growth.

This experiment demonstrates that the higher efficacy and the lower peripheral toxicity of Q_2^{14}gL over the cytotoxic radical Q_2 is attributable to the covalent conjugation of the cytotoxic radical to the targeting carrier LH-RH analog.

Table 26-1

Effect of cytotoxic LH-RH analogs on growth of estrogen independent MXT mouse mammary cancers and survival of mice with Tumors

No	Treatment	Dose (µg/day)	No. of mice	Mean tumor volume in mm^3 on days			Mean survival after transplantation (days)
				10	14	18	
1	Control		15	253	1391	4794	23.1
2	Q_2^{14}gL	68.7	10	33	16	23	28.3 *
3	Q_2	21.3	10	153	144	137	17.9
4	[D-Lys^6]LH-RH	48.0	10	165	1348	4003	23.5
5	[D-Lys^6]LH-RH + Q_2	48.0 + 21.3	10	121	80	27	18.5

All daily doses are 30 nmol equimolar amounts.

Significantly shorter than control ($p<0.05$)

* Significantly longer than control ($p<0.01$) with Duncan's test

EXAMPLE 27

Effects of 2-pyrrolino-DOX (Q_6) and cytotoxic LH-RH agonist analog Q_6^{14}gL ([D-Lys^6]LH-RH linked to Q_6^{14}-O-hemiglutarate) on the growth of androgen dependent rat Dunning R-3327-H prostate carcinomas

Male Copenhagen rats bearing hormone-dependent Dunning R-3327-H prostate carcinomas were treated with Q_6^{14}gL, a new cytotoxic analog of luteinizing hormone-releasing hormone (LH-RH) consisting of the agonist [D-Lys^6]LH-RH linked to 2-pyrrolinodoxorubicin. In the first experiment, 2-pyrrolinodoxorubicin was administered at a concentration of 50 nmol/kg, as a single drug (Q_6) and as an unconjugated mixture with [D-Lys^6]LH-RH or conjugated to the carrier [D-Lys^6]LH-RH (Q_6^{14}gL). Following the second administration of 50 nmol/kg of radical Q_6 alone or mixed with [D-Lys^6]LH-RH, all rats died with signs of general toxicity, whereas all animals, treated with the cytotoxic LH-RH conjugate Q_6^{14}gL, survived. After 5 weeks of treatment with a total dose of 150 nmol/kg Q_6^{14}gL, the tumors regressed from an original volume of 8.35 ±1.7 cm3 at the beginning of the experiment to 4.47 ± 0.8 cm^3, while tumors in the control group continued to grow and measured 17.84 ± 2.2 cm^3. The therapy with Q_6^{14}gL also significantly reduced tumor weight and tumor burden. In the second experiment, designed for comparing the efficacy and toxicity of Q_6 and Q_6^{14}gL the therapeutic regimen consisted of 3 applications of 25 nmol/kg Q_6 or 25 nmol/kg and 50 nmol/kg Q_6^{14}gL. When the treatment was started, tumor volume in all groups was between 3.9 and 4.5 cm^3. After 5 weeks of therapy, the tumors in rats treated with 50 nmol/kg Q_6^{14}gL regressed to 2.3 ±0.51 cm^3 , whereas 25 nmol/kg Q_6 was still toxic and could only produce a reduction in final tumor volume to 6.76 ± 1.4 cm^3, similar to that obtained with 25 nmol/kg Q_6^{14}gL (6.74 ± 1 cm^3), as compared to 15.6 ± 2.2 cm^3 for untreated animals. Histological evaluation of the specimens showed a significant decrease of mitotic cells in the Q_6^{14}gL

treated groups only. LH-RH receptors with high binding capacity were detected in the membranes of untreated Dunning tumor specimens, but after treatment with Q_6^{14}gL, no binding sites for LH-RH could be found. Inhibition of tumor growth by AN-201 and Q_6^{14}gL was also associated with a significant decrease in binding capacity of EGF receptors. As is demonstrated by Figures 4-6, targeted cytotoxic LH-RH analog Q_6^{14}gL is an effective antitumor agent causing regression of rat Dunning R-3327-H prostate carcinomas. Our studies also show that the cytotoxic LH-RH analog Q_6^{14}gL is much less toxic than the antineoplastic radical (Q_6) incorporated, and significantly more active in inhibiting tumor growth.

Figure legends for EXAMPLE 27:

Fig.4. Experiment I: Tumor volume in male Copenhagen rats bearing rat Dunning R-3327-H prostate carcinoma transplants during the treatment consisting of 3 applications of 50 nmol/kg agonist [D-Lys^6]LH-RH and 50 nmol/kg of cytotoxic LH-RH analog Q_6^{14}gL. Vertical lines indicate the SEM. * $p<0.05$; ** $p<0.01$ versus control by Duncan's new multiple range test. The treatment indicated by arrows was applied on days 1,8 and 29.
† The animals treated with Q_6 as a single drug or an unconjugated mixture with [D-Lys^6]LH-RH died in the second week. In these two groups the volume of tumors recorded on day 8 is shown.

Fig. 5. Experiment II: Effect of treatment with 25 nmol/kg 2-pyrrolinodoxorubicin (Q_6), 25 nmol/kg and 50 nmol/kg cytotoxic LH-RH analog Q_6^{14}gL on the tumor volume in rats with Dunning R-3327-H prostate cancer. Vertical lines indicate the SEM. * $p<0.05$; ** $p<0.01$ versus control. The treatment indicated by arrows was applied 3 times, that is on days 1, 8 and 29.

Fig. 6. Experiment II: Effect of treatment with 25 nmol/kg 2-pyrrolinodoxorubicin (Q_6), 25 nmol/kg and 50 nmol/kg cytotoxic LH-RH analog Q_6^{14}gL on the body weight of Copenhagen rats bearing Dunning R-3327-H prostate cancer. Vertical lines indicate the SEM. * $p<0.05$; ** $p<0.01$ versus control. The treatment indicated by arrows was applied 3 times, that is days 1, 8 and 29.

EXAMPLE 28

Comparative study on the effects of doxorubicin (DOX) and targeted cytotoxic LH-RH agonist analog Q_1^{14}gL ([D-Lys^6]LH-RH linked to DOX^{14}-O-hemiglutarate) on the growth of OV-1063 human ovarian carcinoma in nude mice

Human epithelial ovarian cancer cell line OV-1063 originated from a metastatic papillary cystadenocarcinoma of the ovary of a 57-year old woman (Horowitz et.al. (1985) Oncology 42, 332-337). Ten million cells of OV-1063 were injected subcutaneously into three nude mice to grow tumors. Pieces of 1 mm^3 of these tumors were transplanted into sixty animals for in vivo growth inhibition studies. The aim of the experiment was to demonstrate that, as a result of the presence of receptors for LH-RH on OV-1063, the cytotoxic conjugate of LH-RH was more effective and less toxic, than DOX, the cytotoxic radical it contained. Thus, the effects of cytotoxic LH-RH conjugate was compared to those of DOX, the mixture of DOX with the carrier molecule, the carrier alone and the untreated control groups. All injections were administered intra peritoneally. The compounds were dissolved in 0.9 % sodium chloride in water (saline).

Mice with an average tumor size of about 15 mm^3 were divided into six groups of nine animals and received the following treatment seven days after tumor transplantation: group 1, saline; group 2, Q_1^{14}gL at a dose of 700

nmol/20g animal; group 3, Q_1^{14}gL at a dose of 413 nmol/20g animal (maximum tolerated dose, MTD for DOX); group 4, DOX at 413 nmol/20g animal (MTD); group 5, mixture of 700 nmol/20g of DOX and 700 nmol/20g of [D-Lys^6]LH-RH; group 6, carrier agonist analog [D-Lys^6]LH-RH at a dose of 700 nmol/20g animal.

Receptor analysis of OV-1063 showed the presence of high affinity binding sites for LH-RH.

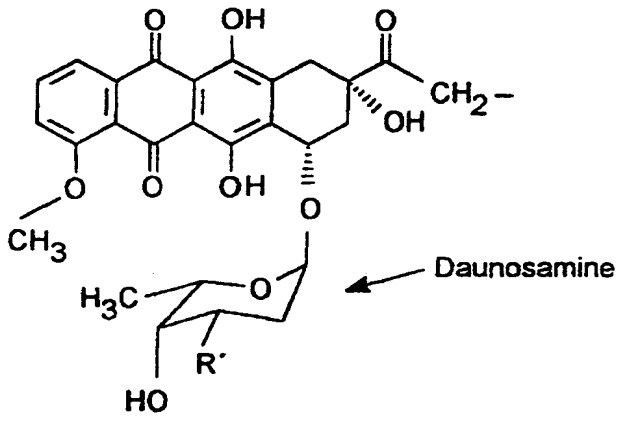
Results: as shown on Fig. 7, strong inhibition of tumor growth was achieved by treatment with Q_1^{14}gL at 413nmol/20g dose (group 3). The animals did not show signs of severe toxicity. In comparison, treatment with DOX administered at the same dose of 413 nmol/20g (12 mg/kg, MTD, group 4) did not result in significant inhibition of tumor growth in the three animals surviving at the end of the expriment. Three animals died by day five and six animals were dead by day nine due to toxicity. At a higher dose, (700 nmol/20g, group 2), Q_1^{14}gL showed very strong inhibition of tumor growth (Fig. 7). Two out of nine animals died due to toxicity and one animal died accidentaly. The six surviving animals were recovering from a weight loss of about 20%at the end of the experiment. In group 6, the same high dose (700 nmol/20g) DOX was mixed with 700 nmol of [D-Lys^6]LH-RH. By day 5, all animals died in this group as a result of severe toxicity.
Conclusions: Our results clearly demonstrate that due to the presence of receptors for LH-RH on the cells of epithelial ovarian cancer OV-1063, targeted cytotoxic LH-RH conjugate Q_1^{14}gL shows lower toxicity and higher antitumoral activity than doxorubicin (Q_1), the cytotoxic radical it contains.

Claims:

1. A compound of the formula:

Q^{14}-O-R-P (I)

wherein Q has the detailed chemical structure:



(II)

where -R- is H or -C(O)-(CH_2)n-C(O)- and n is 0-7

R' is selected from the group consisting of NH_2, an aromatic or hydrogenated 5 or 6 membered heterocycle having at least one ring nitrogen and such a heterocycle having a butadiene moiety bonded to adjacent carbon atoms of said ring to form a bicyclic system and

P is H or a peptide, provided that where R' is NH_2 then R and P are other than H and where R and P are H, then R' is other than NH_2.

2. The compound of claim 1 wherein R' is selected from the group consisting of NH2 , pyrrolidine-1-yl, isoindoline-2-yl, 3-pyrroline-1-yl, 3-pyrrolidone-1-yl, 2-pyrroline-1-yl, 3-piperidone-1-yl, 1,3-tetrahydro-pyridine-1-yl, and

P is P_1, P_2, and P_3,

where P_1 is selected from the group consisting of an LH-RH analog of the formula

Aaa-Bbb-Ccc-Ser-Tyr-D-Lys(Xxx)-Leu-Arg-Pro-Ddd

wherein (Xxx) is hydrogen, A_2Bu or A_2Pr

wherein where:

Aaa is Glp, then Bbb is His Ccc is Trp and Ddd is Gly-NH_2,

where Aaa is Ac-D-Nal(2), then Bbb is D-Phe(4Cl), Ccc is D-Pal(3), D-Trp and Ddd is D-Ala-NH_2, and

where Aaa-Bbb-Ccc is Ac, then Ddd is -NH-CH_2-CH_3

wherein the group Q^{14}-O-R- forms a carboxamido link with the free amino group of the D-Lys moiety or with at least one of the free amino groups of A_2Bu or A_2Pr when present at (Xxx),

P_2 is an analog of somatostatin of the formula

Aaa-Cys-Bbb-D-Trp-Lys-Ccc-Cys-Ddd-NH_2

wherein where:

Aaa is D-Phe, then Bbb is Tyr, Ccc is Val and Ddd is Thr or Trp, where Aaa is D-Trp, then Bbb is Phe, Ccc and Ddd are Thr,

wherein the group Q^{14}-O-R- forms a carboxamido link with the terminal amino group of the Aaa moiety,

P_3 is a bombesin antagonist analog of the formula .

Aaa-Gln-Trp-Ala-Val-Gly-His-Leu Bbb-NH_2

where Aaa is nil, D-Tpi or D-Phe, Bbb is (CH_2-NH)Leu, (CH_2-NH)Phe or (CH_2-NH)Trp or (CH_2-N)Tac

wherein the group Q^{14}-O-R- forms a carboxamido link with the terminal amino group of the Aaa moiety where present or with that of Gln where it is absent.

3. The compound of Claim 2 where n=3.

4. The compound of Claim 3 where R' is NH_2

5. The compound of Claim 3 where R' is 2-pyrroline-1-yl.

6. The compound of Claim 4 where P is P_1.

7. The compound of Claim 5 where P is P_1.

8. The compound of Claim 4 where P is P_2.

9. The compound of Claim 5 where P is P_2.

10. The compound of Claim 4 where P is P_3.

11. The compound of Claim 5 where P is P_3.

12. The compound of Claim 1 where -R and -P are both -H and R' is other than NH_2.

13. The compound of Claim 12 where -R' is pyrrolidine-1-yl.

14. The compound of Claim 12 where -R' is isoindoline-2-yl.

15. The compound of Claim 12 where -R' is 3-pyrroline-1-yl.

16. The compound of Claim 12 where -R' is 3-pyrrolidone-1-yl.

17. The compound of Claim 12 where -R' is 2-pyrroline-1-yl.

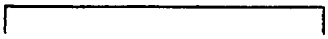
18. The compound of Claim 12 where -R' is 3-piperidone-1-yl.

19. The compound of Claim 12 where -R' is 1,3-tetrahydropyridine-1-yl.

20. The compound of claim 1 of the formula
Glp-His-Trp-Ser-Tyr-D-Lys(Q_1^{14}-O-glt)-Arg-Leu-Pro-Gly-NH_2
wherein Q_1^{14} is doxorubicin-14-yl.

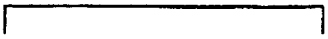
21. The compound of claim 1 of the formula
Glp-His-Trp-Ser-Tyr-D-Lys(Q_6^{14}-O-glt)-Arg-Leu-Pro-Gly-NH_2
wherein Q_6^{14} is 3'-deamino-3'-(2"-pyrroline-1"-yl)-doxorubicin-14-yl.

22. The compound of claim 1 of the formula



Q_1^{14}-O-glt-D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr- NH_2
wherein Q_1^{14} is doxorubicin-14-yl.

23. The compound of claim 1 of the formula

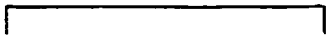


Q_6^{14}-O-glt-D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2;
wherein Q_6^{14}Q614 is 3'-deamino-3'-(2"-pyrroline-1"-yl)-doxorubicin -14-yl.

24. The compound of claim 1 of the formula

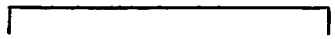
Q_1^{14}-O-glt-D-Trp-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-NH_2;
wherein Q_1^{14} is doxorubicin-14-yl.

25. The compound of claim 1 of the formula



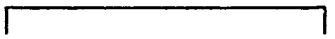
Q_6^{14}-O-glt-D-Trp-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-NH_2
wherein Q_6^{14} is 3'-deamino-3'-(2"-pyrroline-1"-yl)-doxorubicin-14-yl.

26.The compound of claim 1 of the formula



Q_1^{14}-O-glt-D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Trp-NH_2
wherein Q_1^{14} is doxorubicin-14-yl.

27. The compound of claim 1 of the formula



Q_6^{14}-O-glt-D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Trp-NH_2
wherein Q_6^{14} is 3'-deamino-3'-(2"-pyrroline-1"-yl)-doxorubicin-14-yl.

28. The compound of claim 1 of the formula
Q_1^{14}-O-glt-Gln-Trp-Ala-Val-Gly-His-Leu (CH_2-NH)Leu-NH_2
wherein Q_1^{14} is doxorubicin-14-yl.

29. The compound of claim 1 of the formula
Q_6^{14}-O-glt-Gln-Trp-Ala-Val-Gly-His-Leu (CH_2-NH)Leu-NH_2
wherein Q_6^{14} is 3'-deamino-3'-(2"-pyrroline-1"-yl)-doxorubicin-14-yl.

30. The compound of claim 1 of the formula
Q_1^{14}-O-glt-D-Tpi-Gln Trp-Ala-Val-Gly-His-Leu (CH_2-NH)Leu-NH_2
wherein Q_1^{14} is doxorubicin-14-yl.

31. The compound of claim 1 of the formula
Q_6^{14}-O-glt-D-Tpi-Gln-Trp-Ala-Val-Gly-His-Leu (CH_2-NH)Leu-NH_2
wherein Q_6^{14} is 3'-deamino-3'-(2"-pyrroline-1"-yl)-doxorubicin-14-yl.

32. A composition comprising a compound of Claim 1 and a pharmaceutically acceptable carrier thereof.

33. A method of treating a cancer in a mammal which comprises administering to a mammal in need of said treatment an effective amount of a compound of Claim 1.

34. The use of the compounds of claims 20 and 21 for the treatment of various human tumors that have receptors for LH-RH including mammary, ovarian, endometrial, prostatic, pancreatic and colon cancers.

35. The use of the compounds of claims 22-27 for the treatment of various human tumors that have receptors for such somatostatin analogs, including mammary, gastric, pancreatic, colorectal, prostatic cancers, small cell and non-small cell lung carcinomas, renal cell carcinoma, osteosarcomas and brain tumors.

36. The use of the compounds of claims 28-31 for the treatment of various human tumors that have receptors for GRP and bombesin-like peptides including mammary, gastric, pancreatic, colorectal, prostatic cancers, small cell and non-small cell lung carcinomas and brain tumors.

37. A process for the conversion of the nitrogen of a primary amino group of an α,β- or α,γ-hydroxy primary amine into the nitrogen of a monounsaturated nitrogen containing heterocyclic compound having between 5 and 6 atoms in the ring which comprises the sequential steps of

a) treating said hydroxy amine with an excess of a haloaldehyde having an aldehyde carbon, a halo bearing carbon and having 2 or 3 moieties between the aldehyde carbon and the halo bearing carbon selected from the group consisting of CH_2, CH_2CH_2 and OCH_2,

b) adding an excess, relative to the hydroxyamine, of an organic base,

c) neutralizing the said base with a weak acid, and

d) treating with a dilute aqueous acid.

38. The process of claim 30 wherein step a) is carried out in an aprotic reaction inert organic solvent.

39. The process of claim 30 wherein step a) is carried out in a polar non hydroxylic reaction inert organic solvent.

40. The process of claim 30 wherein the solvent is dimethyl formamide.

41. The process of claim 30 wherein the aldehyde is selected from the group consisting of omega-bromo- and omega-iodo-butyraldehyde and valeraldehyde.

Figure 1

Volume changes of estrogen independent MXT mouse mammary cancers in KS-29

(Numbers in italics show surviving mice at the time of measurement)

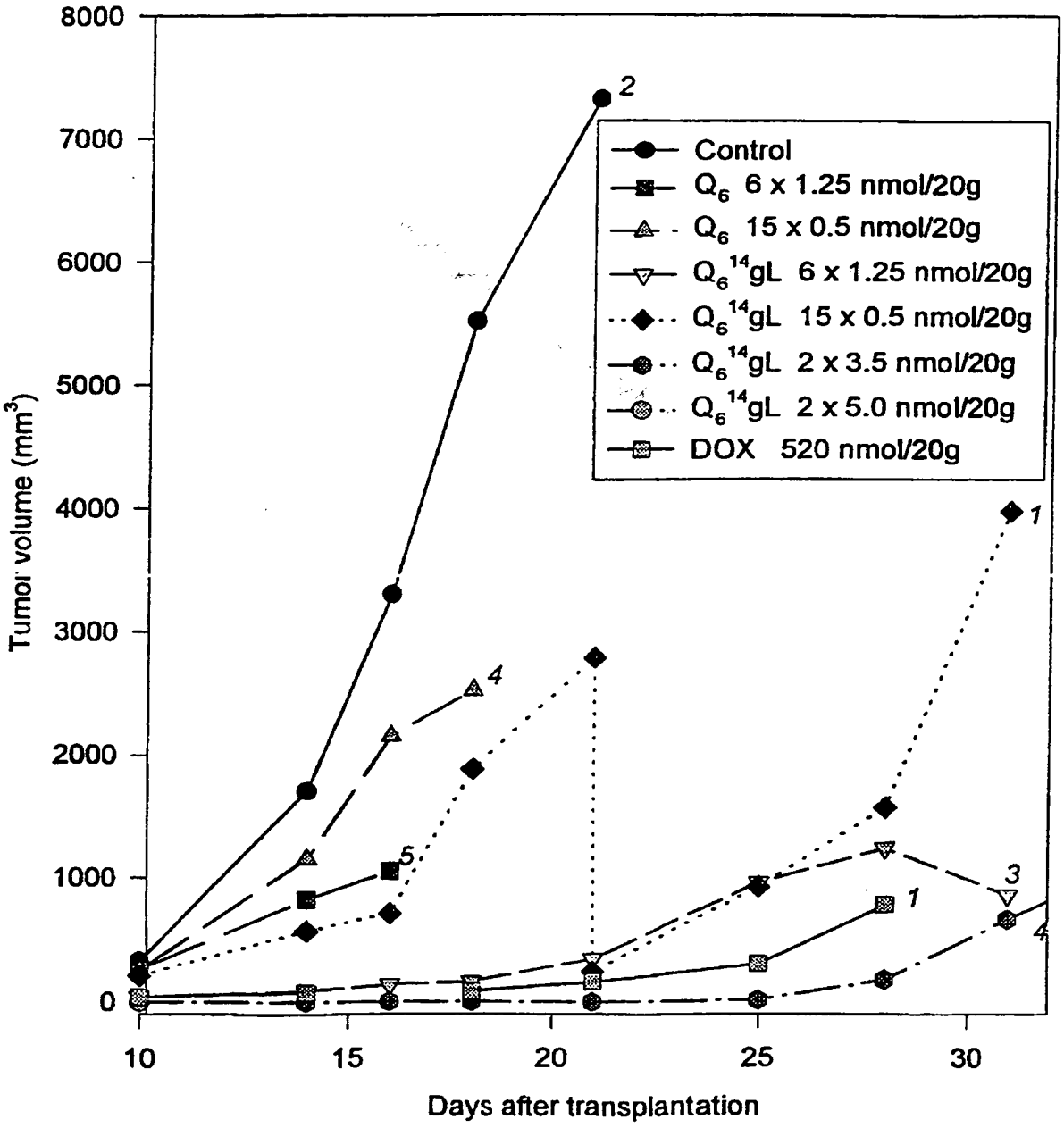


Figure 2

Volume changes of estrogen independent MXT tumors in KS-55 (selected Groups)

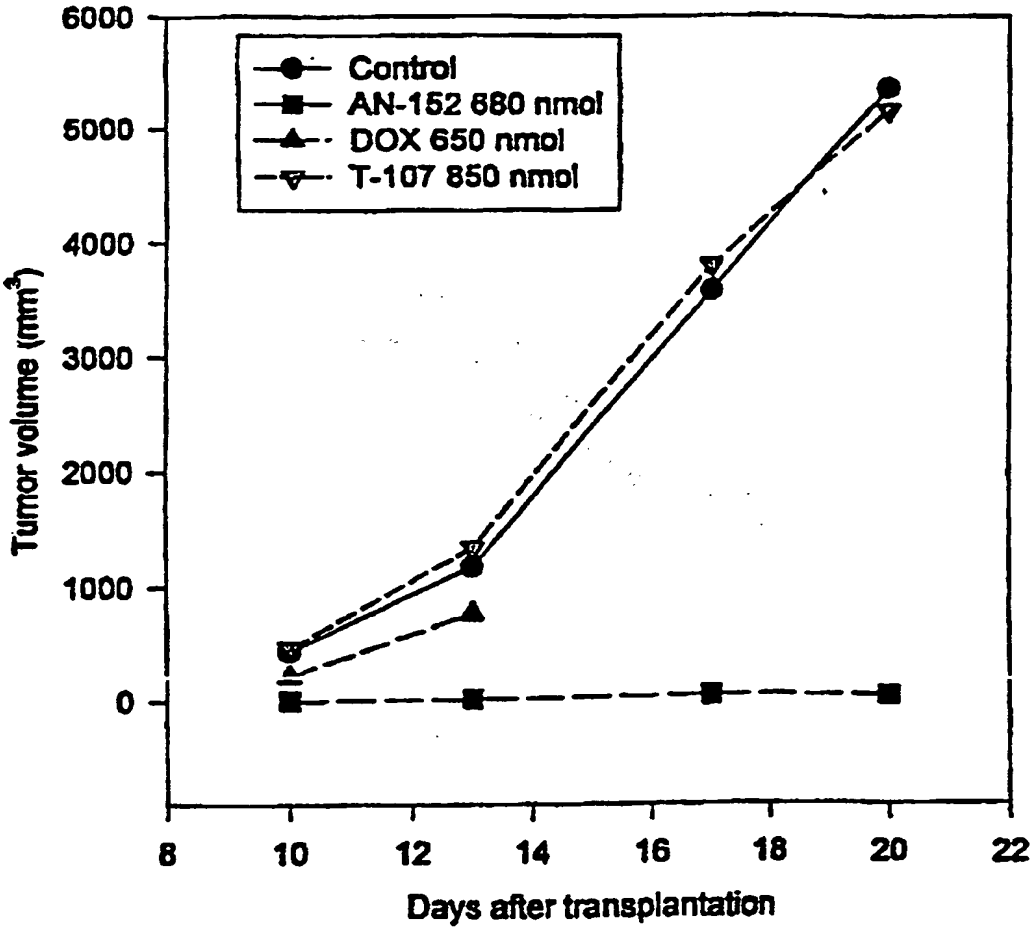


Figure 3 3/7

Effect of cytotoxic LH-RH analogs on survival of mice with estrogen independent MXT cancers

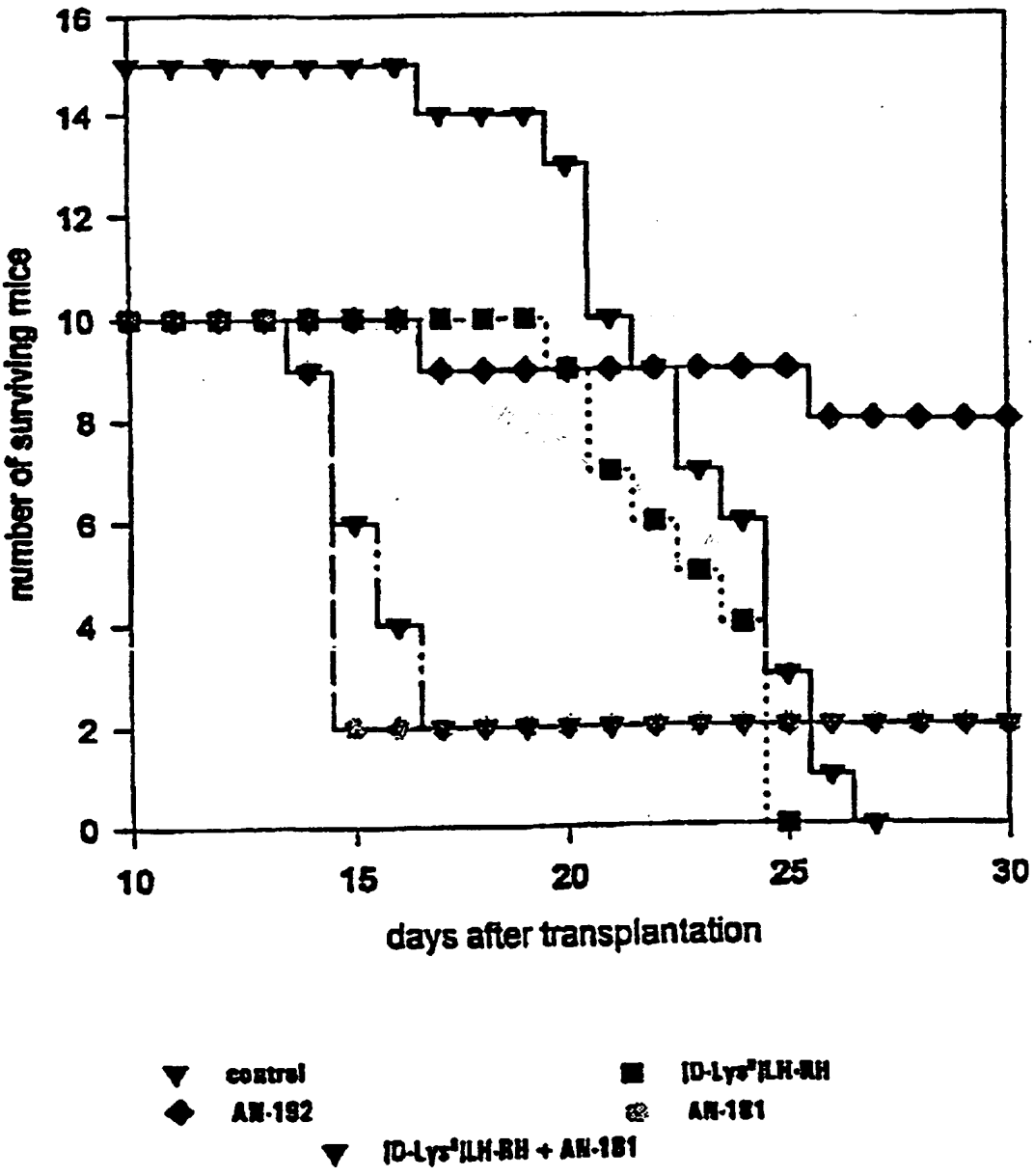


Figure 4

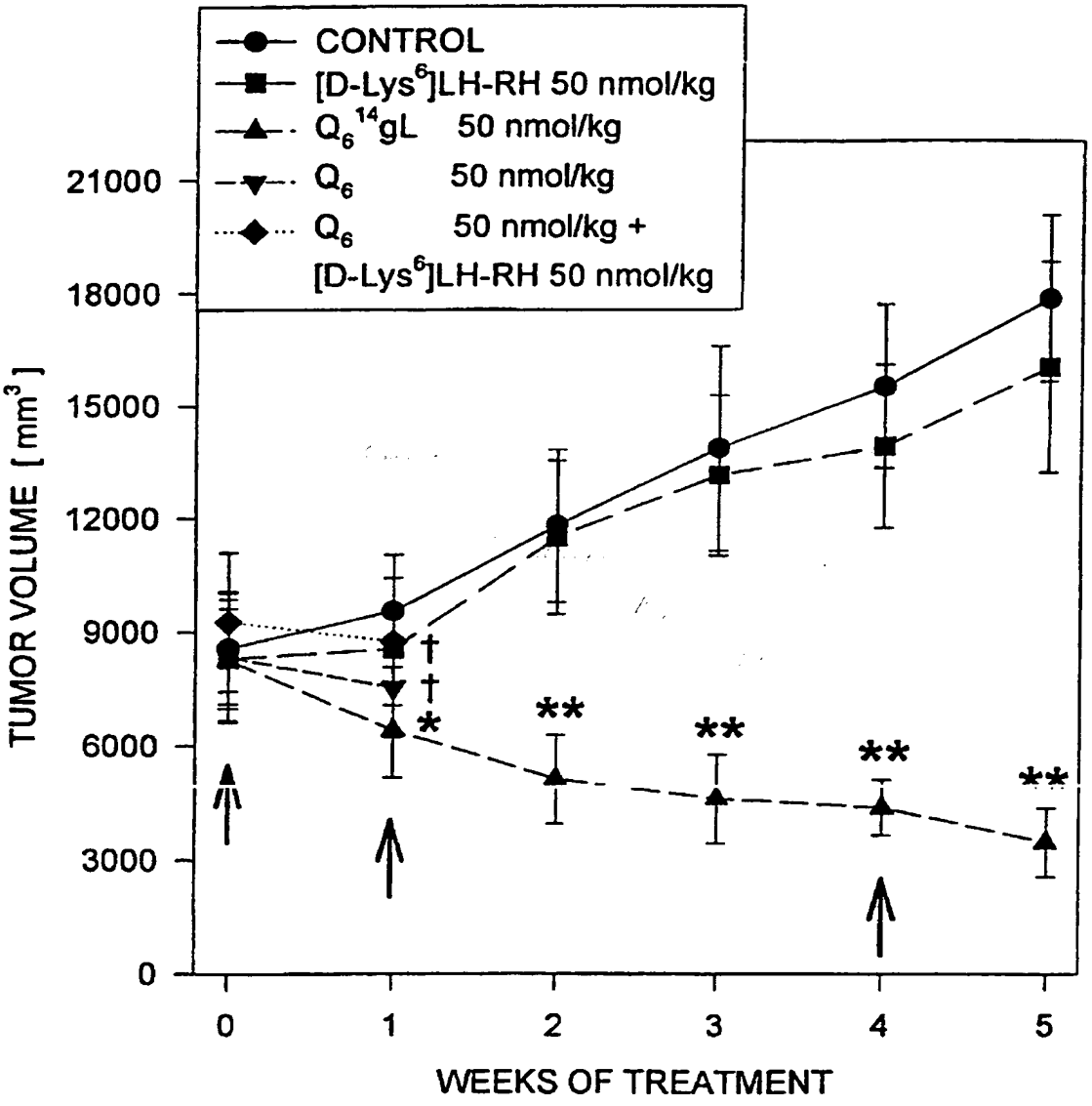

CONTROL
[D-Lys6]LH-RH 50 nmol/kg
Q6 14gL 50 nmol/kg
Q6 50 nmol/kg
Q6 50 nmol/kg +
[D-Lys6]LH-RH 50 nmol/kg
TUMOR VOLUME [mm3]
21000
18000
15000
12000
9000
6000
3000
0
0
1
2
3
4
5
WEEKS OF TREATMENT
†
†
*
**
**
**
**

Figure 5

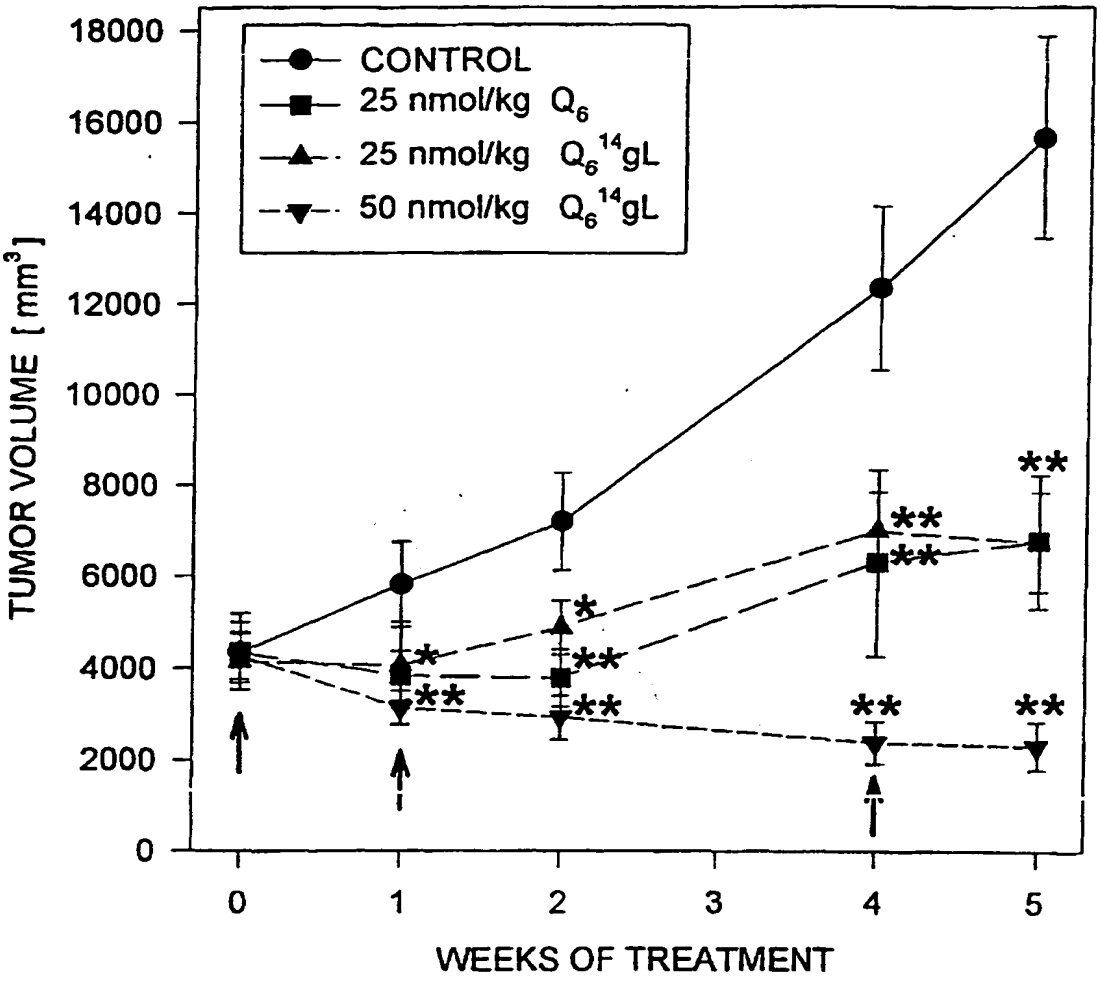

CONTROL
25 nmol/kg Q6
25 nmol/kg Q6 14gL
50 nmol/kg Q6 14gL
TUMOR VOLUME [mm3]
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
0
1
2
3
4
5
WEEKS OF TREATMENT
*
**

Figure 6

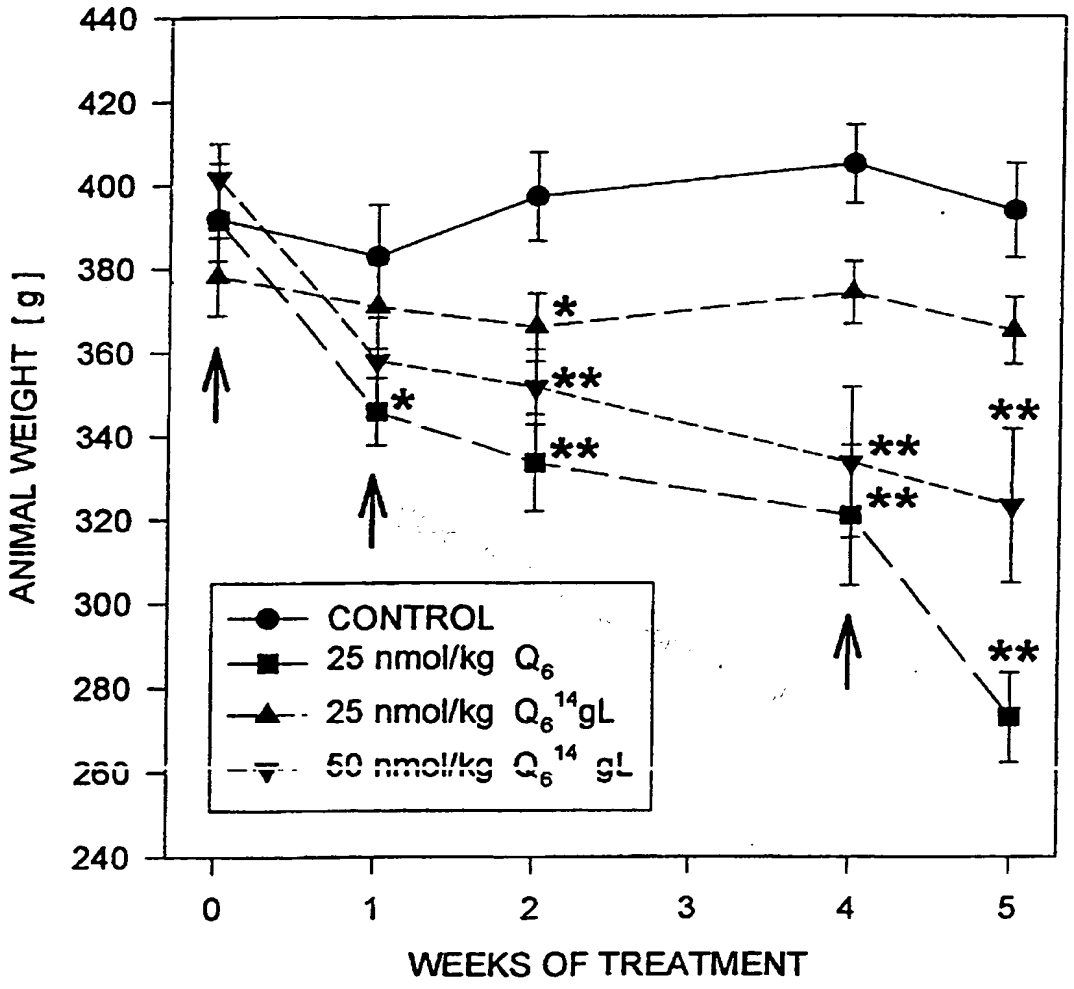
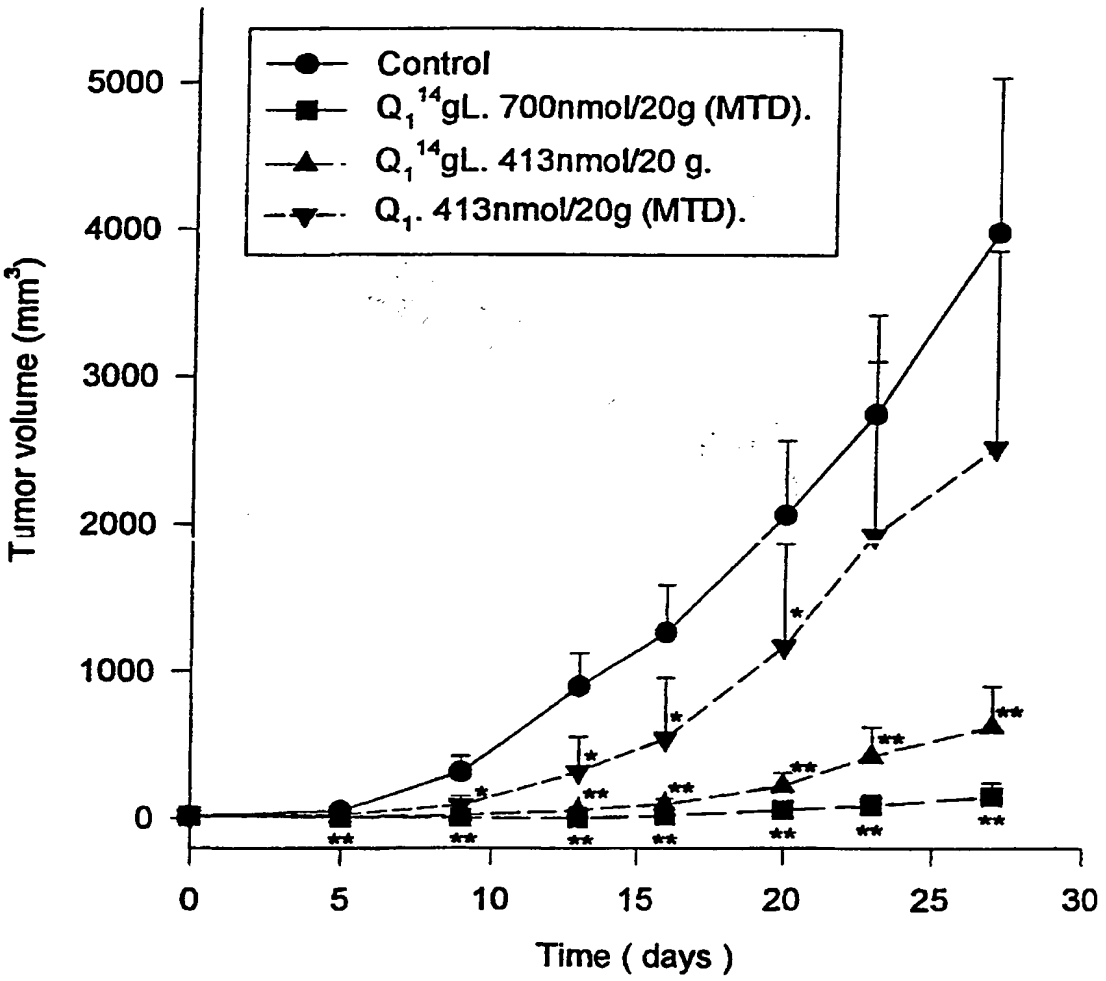

440
420
400
380
360
340
320
300
280
260
240
ANIMAL WEIGHT [g]
0
1
2
3
4
5
WEEKS OF TREATMENT
CONTROL
25 nmol/kg Q6
25 nmol/kg Q6 14gL
50 nmol/kg Q6 14 gL
*
**

Figure 7

Inhibition of growth of OV 1063 human ovarian cancer xenografts in nude mice by cytotoxic analog of LH-RH containing doxorubicin ($Q_1{}^{14}gL$) and doxorubicin (Q_1)



* $p < .05$, ** $p < .01$

MTD ; Maximum tolerated dose

INTERNATIONAL SEARCH REPORT

In tional Application No
PCT/EP 96/05029

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K7/23 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 450 461 A (SCHALLY ANDREW VICTOR ;JANAKY TAMAS (HU); JUHASZ ATILLA (HU); BAJU) 9 October 1991 cited in the application see the whole document ---	1,2, 32-36
A	EP 0 624 377 A (SQUIBB BRISTOL MYERS CO) 17 November 1994 reaction schemes 12, 15, 16, 18 see examples 8,9,14,15,23 see examples 24,28,48,49 see claims; examples 61,70,71 ---	1,32,33
X	US 4 299 822 A (ISRAEL MERVYN ET AL) 10 November 1981 cited in the application see claims; examples --- -/--	1,32,33

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 April 1997	29.04.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Fuhr, C

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Ir tional Application No
PCT/EP 96/05029

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 434 960 A (ERBA CARLO SPA) 3 July 1991 cited in the application see claims; examples	1,32,33
X	GB 2 124 224 A (STANFORD RES INST INT) 15 February 1984 see claims; examples	1,32,33
X	JOURNAL OF MEDICINAL CHEMISTRY, vol. 35, no. 17, 21 August 1992, WASHINGTON US, pages 3208-3214, XP002029565 A. CHERIF AND D. FARQUHAR: "N-(5,5-Diacetoxypent-1-yl)doxorubicin: A New Intensely Potent Doxorubicin Analogue" cited in the application see chart I, Scheme I, scheme III see page 3210, right-hand column, paragraph 4 - page 3212, left-hand column, paragraph 3	1,32,33
X	BIOCONJUGATE CHEMISTRY, vol. 1, no. 5, 1 September 1990, pages 325-330, XP000174625 MUELLER B M ET AL: "ANTIBODY CONJUGATES WITH MORPHOLINODOXORUBICIN AND ACID-CLEAVABLE LINKERS" cited in the application see scheme I and scheme II see page 327, left-hand column, paragraph 1 - page 329, left-hand column, paragraph 2	1,32,33
X	EUROPEAN JOURNAL OF CANCER AND CLINICAL ONCOLOGY, vol. 20, no. 3, March 1984, pages 421-425, XP000670366 F. ZUNINO ET AL.: "Comparison of Antitumor Effects of Aunorubicin Covalently Linked to Poly-L-Amino Acid Carriers" cited in the application see page 421, right-hand column, paragraph 3 - page 422, left-hand column, paragraph 1 see page 424, left-hand column, paragraph 2 - page 425, left-hand column, paragraph 1	1,32,33

-/--

INTERNATIONAL SEARCH REPORT

In tional Application No
PCT/EP 96/05029

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ANTI-CANCER DRUGS, vol. 5, no. 2, April 1994, pages 115-130, XP000670356 A.V. SCHALLY: "Hypothalamic Hormones: from neuroendocrinology to cancer therapy" cited in the application see page 120, right-hand column, paragraph 2 - page 121, left-hand column, paragraph 1 ---	1,2,32, 33
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 79, January 1982, WASHINGTON US, pages 626-629, XP002029566 A. TROUET ET AL.: "A covalent linkage between daunorubicin and proteins that is stable in serum and reversible by lysosomal hydrolases, as required for a lysosomotropic drug-carrier conjugate. In vitro and in vivo studies" cited in the application see figure 1 -----	1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 96/05029

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 33-36
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 33-36 is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

In tional Application No
PCT/EP 96/05029

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0450461 A	09-10-91	AT 127476 T	15-09-95
		AU 638319 B	24-06-93
		AU 7410691 A	10-10-91
		CA 2039908 A	07-10-91
		DE 69112684 D	12-10-95
		DE 69112684 T	01-02-96
		ES 2076393 T	01-11-95
		IE 63278 B	05-04-95
		JP 4224600 A	13-08-92
		WO 9222322 A	23-12-92
EP 0624377 A	17-11-94	AU 6302694 A	17-11-94
		CA 2123363 A	15-11-94
		CN 1100426 A	22-03-95
		CZ 9401187 A	16-11-94
		FI 942237 A	15-11-94
		HU 66485 A	28-11-94
		JP 7070175 A	14-03-95
		NO 941819 A	15-11-94
		NZ 260512 A	25-09-96
US 4299822 A	10-11-81	CA 1150245 A	19-07-83
		CH 645387 A	28-09-84
		DE 3122689 A	06-05-82
		FR 2483928 A	11-12-81
		GB 2077265 A,B	16-12-81
		JP 1458104 C	09-09-88
		JP 57067596 A	24-04-82
		JP 63002440 B	19-01-88
		NL 8102730 A,C	04-01-82
EP 0434960 A	03-07-91	AT 143023 T	15-10-96
		AU 636429 B	29-04-93
		AU 6919491 A	18-07-91
		CA 2046857 A	20-06-91
		CN 1053238 A,B	24-07-91
		CN 1079215 A	08-12-93
		DE 69028610 D	24-10-96
		DE 69028610 T	06-02-97
		WO 9109046 A	27-06-91

INTERNATIONAL SEARCH REPORT

Information on patent family members

In tional Application No
PCT/EP 96/05029

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0434960 A		ES 2094136 T	16-01-97
		FI 97389 B	30-08-96
		HR 921226 A	31-12-95
		HU 209433 B	28-06-94
		IL 96643 A	24-01-95
		JP 4503679 T	02-07-92
		NO 175533 B	18-07-94
		NO 940831 A,B,	14-08-91
		US 5304687 A	19-04-94
GB 2124224 A	15-02-84	US 4464529 A	07-08-84
		AU 1694683 A	26-01-84
		BE 897312 A	18-01-84
		CA 1239396 A	19-07-88
		CA 1233816 C	08-03-88
		CH 655729 A	15-05-86
		DE 3325816 A	26-01-84
		FR 2530640 A	27-01-84
		JP 1606247 C	31-05-91
		JP 2032260 B	19-07-90
		JP 59033278 A	23-02-84
		NL 8302586 A,C	16-02-84
		SE 459921 B	21-08-89
		SE 8304045 A	21-01-84
		US 4826964 A	02-05-89
		US 4585859 A	29-04-86